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Some Aspects of Nitrogen and Energy Metabolism in Cancerous Subjects: *A Review**

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Neoplasia introduces into the body a new race of cells derived from tissues common to ontogeny but differing biologically, structurally, and chemically from their ancestors. The new members of the community of organs and tissues intrude themselves on the nicely balanced economy of the organism. Adjustments are necessary. The stresses imposed by the new growth often transcend the power of homeostatic mechanisms to maintain the host's integrity.

Many disease processes, neoplastic or otherwise, kill by producing anatomic lesions that are incompatible with life. Unrelieved obstruction of a hollow viscus or essential pathway, ulceration with overwhelming sepsis or exsanguinating hemorrhage are lethal regardless of their etiology. A few neoplasms secrete biologically active substances either identical with or similar to naturally occurring hormones. The intense pharmacologic responses elicited ultimately threaten the existence of the host. Some neoplastic and related diseases kill without producing either of these effects. A primary anatomic lesion has often been eradicated by effective therapy, but discontinuous dissemination of tumor cells through the body is found at necropsy. The structural defects produced by the metastases are seldom of sufficient magnitude to threaten life solely for anatomic reasons. Extensive involvement of the liver, for instance, is rarely associated with a syndrome characteristic of hepatic insufficiency. If the same lesions were composed of scar tissue rather than neoplastic cells, the affected individuals might not only be alive but in reasonably good health. The mere presence of an adequate concentration of growing neoplastic cells within the body may cause death. Such cells are cancerous, and their deleterious effects must be described in terms of pathologic physiology.

Pronounced avidity of cancerous tissue for amino acids reflects its remarkable capacity for protein synthesis. Isotopically labeled glycine (23, 40, 51, 64), tyrosine (48, 62), or methionine (10, 31) administered orally or parenterally is taken up by various neoplasms in concentrations approximating those found in the normal structures known to be intimately concerned with protein metabolism. Some investigators have demonstrated that the labeled compounds have been incorporated into tumor protein. The concentration of an isotope in the tumor declines less rapidly with time than it does in normal tissues (23, 51). Reduction in specific activity does not necessarily indicate establishment of an equilibrium of isotope between the cancer and other tissues. A progressively growing neoplasm, unlike the normal structures of the adult organism, increases its mass constantly through continuous manufacture of protoplasm. Anabolism exceeds catabolism. The amount of tagged protein formed by the tumor will depend in some measure on the concentration of labeled amino acid present in its substrate. The specific activity of the plasma reaches a maximum level, then falls progressively with time. Contribution of nonisotopic amino acids from body

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stores or dietary sources probably dilutes the specific amino acids being studied. If the rate of protein synthesis is reasonably constant in the neoplasm, its concentration of isotope necessarily decreases, yet the total amount may increase steadily as long as tagged precursors remain in the blood. Data on the relation of time to the total labeled amino acid in neoplastic tissue, both protein and nonprotein, may yield valuable information. How much exchange exists between the neoplasm and its host? Is the nitrogen metabolism of the cancer essentially a one-way passage? Is the necrotic material in a tumor simply dead and inert, or do split products gain access to the blood stream? Tumors in which the vascular bed is essentially circumferential and those penetrated by a rich network of blood vessels might give different results. The answers to these questions seem to lie within the realm of isotope research, probably cannot be acquired by simpler technics, and are important in formulating a better concept of the tumor-host relationship. An excellent discussion of the use of isotope technics in the study of the protein metabolism of tumors and normal tissues has been published recently by Zamecnik (63).

What is the ultimate source of building blocks used by the growing cancer? The immediate source must be the blood plasma, the composition of which the body attempts to maintain within fairly narrow limits. The principal constituent of neoplastic tissue other than water seems to be protein. Therefore, a study of nitrogen metabolism may be expected to reflect the source of tumor protein. Cramer and Pringle (15) attacked this problem in 1910 by studying the nitrogen balance of rats during the first 2 weeks of growth of the Jensen sarcoma. "In our experiments," they wrote, "the cells of the new growth derived their nitrogenous materal necessary for the building up of new tissue by a sparing action on the protein metabolism. The tumor cells do not proliferate at the expense of the tissues of the host nor is there any evidence that they have a higher affinity for nutritive material than the growing cells of the host." The authors recognized that this physiologic state was replaced later in the course of development of the Jensen sarcoma by pathologic effects which they did not describe. The "sparing action on protein metabolism" that Cramer and Pringle postulated refers principally to a greater increment in mass per unit of nitrogen stored, reflecting the considerably lower concentration of nitrogen and higher water content of the neoplasm they studied (16). Their meager data on nitrogen retention only suggest that the cancerous rats actually stored more nitrogen than the normal rats that ate the same amount of food.

Several observers reported that various transplanted rodent tumors grew at the expense of host tissue, as shown by progressive increase in tumor mass, while carcass weight (animal minus tumor) declined (6, 32, 33, 38, 39). The specific constituents of the carcass tissues that were lost during tumor growth were not defined. Some malignant tumors possess an extremely high priority for building blocks. F. R. White (61) proved conclusively that a transplantable mouse mammary adenocarcinoma could obtain enough nitrogen for its growth from the normal tissues of its host when the diet contained almost no nitrogenous substances. The Walker rat carcinoma 256 grew, although at a diminished rate, even though a strongly negative nitrogen balance was induced by injection of cortisone acetate (29).

It was shown subsequently that large specimens of the Walker carcinoma contained more nitrogen than was stored by their hosts during the course of tumor growth when they ate freely a semi-synthetic diet adequate for growth, pregnancy, and lactation in normal rats (37). Comparable data, listed in Table 1, were obtained from a study of the Murphy-Sturm transplantable rat lymphoma. The contribution of ingested nitrogen to the metabolic pool may be adequate to provide protein building blocks for this neoplasm when it comprises up to 10 per cent of the total body weight (rat plus tumor), but larger lymphomas contain more nitrogen than could have been derived from dietary sources. A similar situation with respect to the Walker carcinoma may be inferred from data reported recently (35).

Sherman *et al.* (52) found that most of the organs and tissues that lost nitrogen during the course of simple caloric starvation also relinquished nitrogen during progressive growth of Walker carcinoma 256. The liver and spleen actually acquired nitrogen, at least temporarily. No consistent significant differences between urinary and fecal excretion of nitrogen by rats bearing Walker carcinoma 256 and pair-fed noncancerous rats of the same age, sex, and initial weight have been observed in this laboratory during the greater part of tumor growth if the cancerous rats ate freely a semi-synthetic diet adequate for growth, pregnancy, and lactation in normal rats. One suspects that the nitrogen lost from body tissues in the cancerous subjects was translocated to the neoplasm. The nitrogen lost from tissues represented only potential building blocks for the cancerous cells, since the methods used measured over-all or net change rather than step-wise alterations.

Norberg and Greenberg (40) injected carboxyl-

labeled glycine-C^{14} intravenously into C3H mice bearing transplanted Gardner lymphosarcoma. The specific activity of the proteins of plasma and various tissues was determined at intervals up to 48 hours and compared with that of the same moieties in noncancerous mice of the same strain. The sarcomatous animals incorporated more isotope into their proteins than did the normal mice at corresponding periods of observation. The uptake was lower in the protein of the wasting muscles of the tumor-bearers than in the muscle protein of normal mice. These differences suggested increased protein turnover among cancerous mice by those parts of the body concerned with protein synthesis and transport, with muscle serving as a main source of protein precursors to supplement dietary intake.

Undoubtedly, some more indolent cancers may obtain adequate supplies of building blocks from ingested materials. Bashford, Murray, and Cramer (7) found that mice bearing some transplanted tumors were able to live for months without visible inconvenience. The weights of the neoplasms often exceeded the weights of their hosts. Emaciation appeared only after ulceration, infection, or hemorrhage complicated the picture. These lesions were certainly cancerous by accepted morphologic and biologic criteria. Variation in behavior characterizes the generality of malignant neoplasms, even those of similar histogenesis.

No information is available on the sources of nitrogen for spontaneous neoplasms in experimental animals, but metabolic studies on a few patients with lymphomatous diseases have been made which provide some insight into the clinical situation. The experiments were based on the principle that an individual fed a constant diet will achieve a dynamic equilibrium among its various compartments. Protoplasm contains nitrogen, phosphorus, and potassium in ratios characteristic of its particular type. Storage or wastage of protein will be associated with retention or excretion of phosphorus and potassium in the proportion in which the three elements exist in the principal tissue or tissues being built or being destroyed. Active osseous metabolism requires that the phosphorus balance be corrected for the phosphorus bound to calcium in bone. Most of the protoplasm of the body is in skeletal muscle. For all practical purposes, the ratio among nitrogen, phosphorus, and potassium found in muscle may be used to indicate changes in protoplasmic mass of the average individual. Albright and other investigators have shown the essential correctness of this thesis, since these elements are excreted or stored in such proportions by relatively normal subjects and those with certain non-neoplastic diseases (4, 20, 49, 58). It should be emphasized that calculations must be made only on a balance basis, the algebraic difference between ingestion and excretion of a particular substance. Any change in the dietary intake may disturb the equilibrium and vitiate the results of the study unless adequate time for establishing a new equilibrium is allowed. The analyses of neoplastic tissue that have been made indicate that most cancers and related tissues contain much more phosphorus and potassium per unit of nitrogen than do normal tissues in general and skeletal muscle in particular. Therefore, a discrete difference among nitrogen-phosphorus and nitrogen-potassium ratios should be expected in the balances of cancerous patients and was actually found in patients with advanced cancer.

Pearson, Eliel, and White (41) studied balances of nitrogen, phosphorus, and potassium in a patient with chronic lymphatic leukemia. Treatment with ACTH not only increased the negative nitrogen balance markedly but caused a disproportionate increase in elimination of phosphorus and potassium which reflected the ratios among nitrogen, phosphorus, and potassium found on analysis of tumor tissue from the same patient. A period of nitrogen retention in the same subject was accom-

TABLE 1

NITROGEN LOST FROM CARCASS DURING TUMOR GROWTH EQUALS NITROGEN IN TUMOR PLUS NET NITROGEN BALANCE
(Murphy-Sturm Lymphoma)

Rat	Tumor weight (per cent)	Change in carcass N (gm.)	Net N balance (gm.)	Tumor nitrogen* (gm.)	Net N balance plus tumor N (gm.)
1	4.6	+0.069	+0.444	−0.404	+0.040
2	8.3	+0.310	+1.052	−0.606	+0.446
3	10.6	−0.329	+0.450	−0.774	−0.324
4	22.7	−1.076	+0.424	−1.595	−1.171
5	24.5	−1.470	+0.037	−1.415	−1.378

*Tumor nitrogen bears a negative sign, since it is not available to the uses of the body under conditions of progressive tumor growth. The net nitrogen balance plus tumor nitrogen (column 6) is the algebraic sum of columns 4 and 5.

panied by an increased storage of the other two elements in the direction of the proportions in which they were found in the neoplasm rather than in muscle.

Fenninger and Waterhouse (21) studied one patient with acute leukemia who ate a constant diet and retained nitrogen for 67 days. The proportions of nitrogen to phosphorus and potassium stored were neither those characteristic of muscle nor of the patient's leukemic cells. They derived an equation to determine the partition of nitrogen between the host and the neoplasm. Application of the formula indicated that all of the ingested nitrogen retained and additional nitrogen derived from the normal tissues were used for tumor formation. The calculated rate of tumor growth correlated well with clinical and autopsy findings. Similar studies in a patient with lymphosarcoma indicated that excess nitrogen excreted prior to therapy was derived mainly from host tissues, though the tumor grew progressively. Induction of a clinical remission with ACTH caused excretion of nitrogen, phosphorus, and potassium in the ratio in which they were found in the subject's neoplasm.

Some clinical cancers or related lesions, then, may derive building blocks from body stores.This again is a net result and does not reflect the actual dynamics of intermediary metabolism. The methods used in these studies seem basically sound but probably can be used only when relatively large quantities of rapidly growing neoplasms are present. The common observation of weight loss in cancer patients whose neoplasms are obviously growing may be further evidence for translocation of protein from normal to neoplastic tissue. The demonstration of negative nitrogen balance in such individuals would permit of no other rational conclusion.

If the growing cancer can derive nitrogenous building blocks from normal tissues, it should be able to draw on more labile body proteins. Parfentjev and Duran-Reynals (43) observed a decrease in Proteus agglutinin of chicken sera throughout the development of Rous sarcoma. Eighty per cent of the sera from people with nonneoplastic diseases agglutinated with Proteus antigen, but only 28 per cent of the sera from cancer patients whose lesions were untreated reacted similarly (42). Diminished antibody titers and associated impairment of immunologic response in patients with leukemia (22) and Hodgkin's disease (19, 57) have been reported. Wharton *et al.* (60) found that rapidly growing neoplasms of mice yielded lower titers of antisera when immunized with various antigens than did similarly treated normal mice. The difference in response was not found after regression of tumors nor in mice that bore slowly growing neoplasms. They also found evidence for an abnormally high destruction of antibody protein in cancerous mice, since passive transfer of immune serum from normal to cancerous mice failed to increase the antibody titer of the tumor-bearing subjects to the same extent as it did in noncancerous animals. Lastrapes[1] studied rabbits that had been immunized against horse serum prior to intratesticular transplantation of the Brown-Pearce carcinoma. Progressive growth of the cancer with or without subsequent regression was accompanied by pronounced reduction in antibody titer. Noncancerous rabbits maintained their precipitins against horse serum at a high level. No difference in the total circulating gamma globulins was detected among the three groups.

Perhaps the cancerous host diverts both antigenic and antibody proteins to serve bodily functions and the demands of tumor growth. A hierarchy exists among the various uses for protein in the animal's economy. Apparently, the priority ratings may be altered in response to poorly defined stresses. The work of Cannon and his colleagues is pertinent (12). Elimination of an essential amino acid from the diet or reduction in food intake to the extent of protein depletion diminished the antibody response to antigenic stimuli. Failure of alimentation frequently accompanies the growth of cancer in man and experimental animals. This factor must be considered in evaluating immunologic responses.

Hypoalbuminemia often accompanies clinical cancer (18, 27, 34, 44, 45, 50). It may be found among those patients in good nutritional state whose neoplasms are small. The degree of hypoalbuminemia increases as cancerous growth progresses. The concentration of plasma albumin falls more rapidly than does the concentration of total plasma proteins due to an absolute increase in globulins, particularly alpha globulins and fibrinogen (34).

A decrease in serum albumin is frequently considered presumptive evidence of depletion of body protein. In those patients with pronounced anorexia and wasting of body tissue, it seems probable that protein depletion has occurred and doubtless has contributed to the production of low plasma albumin. Diminished food intake need not be the only mechanism by which hypoalbuminemia is produced in cancerous patients. On theoretical grounds, at least, the translocation of nitrogen from general body stores to form neoplastic tissue might bring about a reduction in plasma

[1] Personal communication.

albumin. On the other hand, the finding of hypoalbuminemia in relatively early cases of cancerous growth suggests that some other mechanism must be operating.

Cuthbertson and Tompsett (17) described a fall in plasma albumin and marked rise in plasma globulins following trauma to dogs. Maximum alterations in the concentrations of plasma proteins preceded the catabolic phase of reaction to injury. Peters (46) observed a rapid decline in serum albumin during the 48 hours immediately following surgical operations. Nitrogen excretion was not large during this period, nor could blood loss, shock, or hemodilution account for the change. While an insult sufficiently severe to initiate the "stress reaction" mediated through the adrenal glands characterized the work of Cuthbertson and Tompsett and of Peters, no such acute episode can be demonstrated in the history of the average cancer patient who has not been subjected to definitive surgery. It is possible, however, that the same or similar mechanism may operate in the absence of an acute stress. Evidence for altered hepatic function in the genesis of hypoalbuminemia is provided by workers who studied certain standard liver function tests in patients with cancer, principally those of gastrointestinal origin (1, 5, 47, 54). They found that a considerable proportion of the tests used gave results that would not be expected in an average, healthy adult. The specificity of various tests of hepatic function is open to considerable question, but it can hardly be denied that the cancerous subjects they studied had some metabolic alteration in which liver dysfunction played a part. Dysfunction is used in the sense of any deviation from the accepted norm rather than connoting a parenchymal disturbance so severe as seriously to limit the capacity of the organ to metabolize the substances studied. The key position that hepatic tissue holds in the body's metabolism would require it to react to various stimuli that threaten the existence of the host. It is not surprising that cancer should be one such stimulus. The mechanism by which hypoalbuminemia is attained in the patient with comparatively early cancer is by no means clear and requires intensive investigation. It is unfortunate that the information currently available on the state of plasma proteins among cancerous subjects is limited to observations of concentration of these substances in the circulating plasma. A good study of the total circulating proteins might add much to our knowledge.

Whereas the dispensable fraction of nitrogen in normal components of the body is available to meet changing demands, that within cancerous cells is lost unless progressive growth of the tumor can be arrested. Large Walker carcinomas which had killed their hosts seldom contained as much nitrogen as was lost from the bodies of rats that died of simple caloric starvation (37). Depletion of normal protein stores doubtless contributes importantly to the deleterious effects that neoplasms produce in their hosts.

Synthesis of protoplasm requires expenditure of energy. Cachexia is a common consequence of cancer both in man and experimental animals, but the mechanism by which this state is attained is difficult to evaluate. Cramer (14) found that the respiratory quotients of rats bearing Jensen sarcoma did not differ from those of normal rats until weight loss occurred in the cancerous host. Thereafter, more carbon dioxide was eliminated per unit of oxygen inspired. Combustion of fat reduces the nonprotein respiratory quotient. Progressive growth of either Walker carcinoma 256 or the Murphy-Sturm lymphoma in rats is accompanied by steadily decreasing food intake. Anorexia does not commence with the inception of tumor growth but apparently starts after the neoplasm has reached a critical size. Rats in which the Walker tumor grew progressively lost significantly more lipid than did pair-fed noncancerous rats of the same age, sex, and initial weight (36). The caloric value of the amount of fat lost equaled the total calories lost from the body during tumor growth as determined by bomb calorimetry (35). It seems clear from carcass analysis of rats bearing the Walker tumor that progressive lipid loss does not begin until the onset of anorexia.

Haven, Bloor, and Randall (24) stated that the proportion of carcass lipids in rats bearing Walker carcinoma 256 varied inversely with the size of the tumor and that the blood lipids were often markedly increased, owing principally to an increase in fatty acids. They showed (25) subsequently that the lipemia reached a peak during the course of cancerous growth, after which interval blood lipids declined to normal values before the death of the subject. Begg and Dickinson (8) noted lipemia in the sera of rats bearing Walker carcinoma 256 which was not present in noncancerous rats fed the same diet. Adams (2) found that CBA mice bearing Gardner lymphosarcoma developed fatty livers if they were fasted for 48 hours. This phenomenon was not observed either during the very early or very late phases of tumor growth. Perhaps lipemia reflects an increased mobilization of lipids to meet increasing energy demands. The rats in which there is a progressive growth of the Walker carcinoma 256 use saturated fatty acids preferentially and seem to hoard the unsaturated forms (25).

The lipid content of the Murphy-Sturm lymphoma, the Walker carcinoma (37), and the Gardner lymphoma (3) also decreases as the neoplasms grow larger. The reason for this is not known. It might conceivably be related to availability of lipid to the tumor, since rats which ingested a diet containing 72 per cent of total calories as fat (Crisco) maintained a constant lipid concentration in their cancers of approximately 2 per cent.

Carbohydrate and fat provide the principal sources of energy for most mammals. The caloric expenditure of these substances by rats bearing the Walker carcinoma was estimated by a combination of chemical analysis of body lipid and bomb calorimetry (36). A constant relationship was suggested between energy expenditure and tumor growth, but the former was independent of growth rate. Further experimentation with more precise technics is required to establish this point.

Weight loss of itself implies excessive expenditure of energy only in relation to dietary intake. Diminished alimentation is accompanied by decrease in basal metabolic rate in average healthy subjects (9, 30). Does the cancerous individual react in the same way to the stress of reduction in food intake? This question cannot be answered categorically. Pertinent data regarding the clinical situation indicate great variation in the basal energy expenditure of the subjects studied. It may be significant, however, that the literature records few instances of significantly lowered basal metabolic rates among patients with extensive cancerous lesions, whereas definitely increased rates are listed frequently (11, 26, 53, 56). Some of the earlier workers believed that an increased energy expenditure in cancerous patients was of grave prognostic import.

The absence of weight loss among many patients whose cancers are amenable to definitive treatment indicates that malignant neoplasms do not increase the caloric requirements of their hosts at all stages in the evolution of the malignant state. Waterhouse, Fenninger, and Keutmann (59) studied eight patients with widely disseminated but different anatomic types of neoplastic or related diseases. They ate diets that should have provided adequate caloric and protein intakes for maintenance of weight in average healthy subjects of similar age and habitus. All except one febrile patient retained nitrogen. Four of the subjects spent more energy as calculated by Newburgh's method than the diets provided. The excessive caloric expenditure was not always reflected by loss of weight, since some patients with advanced cancer tended to store large quantities of water. These observations require confirmation, preferably by some other method for measuring caloric output.

Circumstantial evidence exists, then, for an increased energy expenditure by some cancerous hosts under poorly defined conditions. It may be useful to speculate on possible reasons for this phenomenon. In the final analysis, the basic mechanisms must involve either a failure of the organism to realize the potential caloric value of its foodstuffs or the initiation of some processes within the body that require expenditure of additional energy or both. The former idea seems rather improbable.

High rates of aerobic and anaerobic glycolysis characterize carbohydrate metabolism of most cancer tissues *in vitro*. There is good evidence that tumors glycolyze rapidly in the body as well. Efferent blood from the tumors studied *in vivo* by the Coris (13) contained less glucose and more lactic acid than did the afferent blood. Voegtlin *et al.* (55) demonstrated a decrease in the pH of the neoplastic tissue following parenteral administration of glucose to its host. From the first law of thermodynamics it follows that the total energy derived from aerobic or anaerobic degradation of glucose to carbon dioxide and water must be the same. This does not mean, however, that all the energy liberated can be used for metabolic processes. Current concepts suggest that high energy phosphate bonds are a major source of metabolic energy. Despite the high lactate content of efferent blood from cancers, no systemic acidosis can be demonstrated. Evidently, the lactate is metabolized by normal tissues, probably chiefly by the liver. If the lactate be oxidized to pyruvate, and the latter burned completely through the tricarboxylic acid cycle, then the full potential value of the parent carbohydrate can be realized. On the other hand, the lactate may be synthesized to glycogen before complete utilization. Such a route would require the investment of high energy bonds to establish lower energy bonds. The extensive use of such an indirect pathway would waste energy. Apparently, the cancerous organism is able to metabolize both carbohydrates and fats completely as far as their end products are concerned. No evidence of ketonuria or of an increase in titratable acidity of the urine was found during uncomplicated neoplastic growth.

Protein metabolism in the cancerous host may conceivably require more energy than in pair-fed normal rats. The nitrogenous constituents of normal tissues that are released into the blood stream can probably not be utilized directly by all the other cells in the body. They first must be built into materials that the cells can metabolize. Native proteins probably must be converted to plasma

proteins, to polypeptides or amino acids if they are to be available for the synthesis of protoplasm. Amino acids may be deaminated in order that their carbon residues may be burned to provide energy. A comparison of nitrogen excretion of rats with Walker carcinoma 256 maintained on an ad libitum diet and that of the noncancerous pair-fed controls of the same age, sex, and initial weight indicates that there is no significant difference in the total excretion of nitrogen until late in the course of tumor growth. In the ante mortem period, during which precipitous weight loss characterizes the tumor-bearing rat, large quantities of urinary nitrogen are excreted as urea and ammonia. At this point the rat has lost almost all its body fat, and probably the heightened excretion of urea and ammonia indicates that amino acids from tissue proteins as well as dietary proteins are being deaminated in order that their residues may furnish needed energy. The specific dynamic action of protein seems to be due principally to cleavage and deamination or to other forms of molecular arrangement of nitrogenous substances. A heightened degradation, then, would be expected to increase specific dynamic action. Strieck and Mulholland (53) found that the ingestion of egg white by three cancer patients whom they studied did not increase the basal metabolic rate. This failure to observe specific dynamic action after protein ingestion deserves further study. It is possible that the cancerous individual at certain stages of tumor growth may be metabolizing proteins and amino acids at a maximum rate even under resting conditions; this fact, in part, may account for excess expenditure of energy.

The metabolic energy of foodstuffs available to the cancerous host will depend upon the relative efficiency of intermediary metabolism.

Obviously, the growing neoplasm utilizes energy. This might be of little or no consequence if a large supply of combustibles were provided through a compensatory increase in appetite. This has not been observed. Anorexia restricts the availability of food for the organism, and the meager amount ingested must serve the needs of both host and cancer. Apparently, a considerable proportion of the energy available to the cancerous organism is utilized by the tumor. Thus, normal structures may be further deprived of energy sources.

Perhaps the foregoing discussion applies only to those cancerous subjects in which food intake is seriously impaired. Begg and Dickinson (8) fed Ingle's high fat diet (28) by gavage to cancerous and noncancerous rats of the same age, sex, and initial weight. The ration provided approximately 40 calories per day. The carcasses of the tumor-bearing animals gained as much weight during growth of Walker carcinoma 256 as did the normal rats. The neoplasms comprised 20–24 per cent of the total weight of the cancerous subjects at the end of the experiment. We have observed a group of rats eating Ingle's high fat diet in which the egg albumin was replaced by an equal amount of lactalbumin to circumvent the possibility that a relatively high intake of avidin might impede tumor growth. The animals received the diet from the time of weaning, and after they reached 100 gm. in weight the daily intake approximated 31 calories. Walker carcinomas commenced to grow when the rats weighed 229 gm. Most of the animals developed anorexia, but two rats maintained their appetites for 5 weeks when the tumors constituted 20 per cent of body weight. The carcasses (rat minus tumor) of the cancerous rats weighed 278 and 282 gm., respectively, while a normal rat of the same age and sex which had grown at the same rate while eating identical quantities of the same diet weighed 325 gm. The failure to achieve as good weight increment in the cancerous carcasses as in the noncancerous rats is doubtless due to the appreciably lower caloric intake provided. The important point to determine is the ultimate pathologic effects of cancerous growth in subjects whose protein and energy requirements are maintained through adequate alimentation. Begg and Dickinson (8) have already shown that anemia, adrenal enlargement, and depression of hepatic catalase activity occurred among their cancerous rats in the absence of weight loss from the carcass. It remains to be shown that the carcasses of the tumor-bearers had the same general composition as did the noncancerous control rats. Perhaps forced feeding merely prolongs the early period of tumor growth so that the onset of the starvation phenomenon is delayed. Other aberrations in the host's physiologic reactions may be equally lethal, for occasional rats ingesting our standard diet in unrestricted amounts maintain a good intake of protein and the principal calorigenic foodstuffs. These animals invariably contain considerable quantities of total body lipid at death and do not exhibit an "ante mortem rise" in urinary nitrogen excretion. Our knowledge of nitrogen and energy metabolism in cancerous hosts is far from complete.

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Liver Nitrogen in Tumor-bearing Rats

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In a previous study of tumor-bearing rats of the Wistar strain (12), it was shown that the growth of a neoplasm elsewhere than in the liver itself was frequently accompanied by an increase in the weight of the liver. It was recognized that the percentage of liver water was increased in such animals (4, 8); but, according to McEwen and Haven (8), the dry livers of rats with subcutaneous implants of carcinosarcoma 256 were not significantly heavier than those of control animals. However, the dry weight may not be a reliable indication of the protein content, inasmuch as Kosterlitz (7) has shown that an alteration in liver nitrogen components may be accompanied by an opposite change in the amount of glycogen or fat or both, with the result that little or no change is detectable in the total dry weight. Recently, a report by Sherman, Morton, and Mider (10) showed that the livers of cancerous rats acquired nitrogen during a part of the period of tumor growth. The present study indicates that, in rats with subcutaneous tumors of varying sizes, (*a*) the nitrogen of the liver increased significantly when large growths were present and (*b*) the degree to which the nitrogen accumulated was proportional to the total mass of the rat—that is, to the weight of the carcass plus the weight of the tumor.

MATERIALS AND METHODS

Wistar albino rats, 22 males and 14 females, 1–1½ years of age, were used. At 30 days of age the males, from the colony of Dr. Margaret Reed Lewis, had received subcutaneous inoculations of methylcholanthrene (Eastman Kodak Co.). Tumors failed to appear in five of these rats (designated below as "tumors 0 weight"); the remainder developed typical fibrosarcomas. The females were obtained from the stock colony of the Wistar Institute and bore spontaneous mammary tumors (fibroadenomas).

Each rat was killed with ether and weighed, and the tumor removed and weighed. All the growths were well encapsulated, and none was necrotic. The liver was dissected out and handled according to the method of Gurd, Vars, and Ravdin (5). After extraneous fat and connective tissue were trimmed away, the lobes were cut apart and gently pressed to expel excess blood. Two slices were cut from the right lobe, placed in weighing bottles, and promptly weighed. The remainder of the liver was also weighed, and samples were taken for histological preparations. The livers appeared to be normal grossly, except for enlargement in animals with heavy tumors. No pathological changes of importance were seen in other organs, and no metastatic growths were observed.

The slices of liver were dried in an oven and desiccator to constant weight. From the total wet liver weight and the water loss of the duplicate samples, the total dry weight was calculated. Nitrogen determinations were made on the dried slices by a semi-micro-Kjeldahl method. The average of the two nitrogen determinations was used to calculate the nitrogen of the whole dry liver.

RESULTS

The data for each rat are recorded in Table 1, in which the males and females are listed in descending order of tumor weight. The table also includes the calculated ratios of liver nitrogen to body weight with and without the tumor. The relationship between the relative liver nitrogen and the weight of the tumor is shown in Chart 1. Values for animals with no tumors are represented as horizontal bars on the zero line.

In the upper portion of the graph it may be seen that, with increasing tumor weight, the amount of nitrogen in the liver also increases beyond the amount to be expected from carcass weight alone. There is some indication that this increase in liver nitrogen is more marked in the female rats, but the number of animals involved is too small to draw such a conclusion. In the lower part of the graph it may be seen that the amount of nitrogen in the liver relative to the weight of the carcass plus the tumor remains constant, whether the tumor be large or small.

Sections of the liver examined under the microscope appeared to be normal.[1] Differences observed

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[1] We are indebted to Dr. John E. Gregory, Professor of Pathology, Hahnemann Medical College and Hospital, for evaluation of sample sections.

in the appearance of the cytoplasm were attributable to variation in glycogen content. The amount and distribution of connective tissue, shown by Mallory and Masson trichrome stains, were alike in all sections. Comparisons of cell size were made by ocular micrometer measurements of the greatest diameter of 500 cells per liver in a number of rats, and nucleocytoplasmic ratios were determined by the method of Chalkley (2). Neither determination showed consistent differences among the rats.

DISCUSSION

The results reported here suggest a linear, direct relationship between the amount of nitrogen in the liver and the total mass of the organism in which the liver functions. In a normal rat the size of the liver (and, more particularly, its protein content) varies with the body size. This relationship has been interpreted to signify an adjustment to the requirements of the animal's tissues (9, 11). If the tissues of the host are augmented by a neoplasm, apparently the liver nitrogen increases, not in an all-or-none fashion, but in proportion to the sum total of the normal and the tumorous tissue.

The mechanism responsible for the enlargement of the liver in these circumstances has not been elucidated. Greenstein (4) implied an impairment in the function of the liver presumably owing to a product of the tumor circulating in the blood stream. Homburger (6) described hyperplasia and increased nitrogen of lymphoid tissue in mice with transplanted tumors, and suggested that the adrenal gland might mediate the effect. Campbell and Kosterlitz (1) implicated placental hormones in the increased turnover of radioactive phosphorus and the greater amounts of desoxy- and ribose-nucleic acids in the livers of pregnant and lactating rats. In addition to the possibility that hormones or tumor products may be responsible for the enlargement of the liver, some part might be played by a lowered level of the plasma proteins, which

TABLE 1

DATA ON INDIVIDUAL RATS

Animal	Tumor wt.	Body wt. (gm.)		Liver wt. (gm.)		Liver nitrogen (mg.)		
		Plus tumor	Minus tumor	wet	dry	Total	mg/100 gm body plus tumor	mg/100 gm body minus tumor
Male no.:	(gm.)							
1	152	448	296	19.23	4.36	514	115	173
2	150	479	329	14.17	3.83	429	90	130
3	135	421	286	11.57	3.09	379	90	132
4	131	456	325	16.07	4.10	484	106	149
5	123	438	315	13.32	3.61	404	92	128
6	122	383	261	11.39	3.05	356	93	136
7	114	416	302	13.35	3.86	443	106	147
8	114	425	311	12.71	3.34	406	96	131
9	89	433	344	11.84	3.49	375	86	109
10	62	383	321	11.27	3.04	351	92	109
11	42	323	281	9.61	2.74	306	95	109
12	37	401	364	11.13	3.40	335	92	84
13	9	357	348	9.00	2.51	295	83	85
14	7	361	354	9.85	2.86	325	90	92
15	7	308	301	8.14	2.00	234	76	78
16	0		404	11.82	3.70	391		97
17	0		329	9.21	2.78	303		92
18	0		382	11.15	3.47	370		96
19	0		326	10.02	3.07	332		102
20	0		306	7.77	2.35	262		86
21	0		379	11.49	3.60	374		99
22	0		392	11.74	3.84	384		98
Female no.:								
1	171	482	311	17.67	4.86	494	102	159
2	115	362	247	12.35	3.42	357	99	145
3	94	408	314	16.79	4.72	490	120	156
4	55	319	264	13.98	3.78	405	127	153
5	52	340	288	12.24	3.23	347	102	120
6	45	303	258	9.97	2.83	294	97	114
7	39	296	257	9.99	2.89	293	99	114
8	38	308	270	11.17	3.18	324	105	120
9	27	314	287	11.20	3.05	300	96	105
10	19	314	295	12.04	3.49	362	115	123
11	18	260	242	9.97	2.84	295	113	122
12	10	295	285	9.97	3.01	295	100	104
13	9	273	264	10.14	3.04	300	110	114
14	6	257	251	8.12	2.19	254	107	101

are known to be provided by synthesis in the liver (3, p. 52). Greenstein (4) stated that blood proteins tend to be lowered in tumor-bearing hosts. Possibly they may be withdrawn from the circulation at an increased rate for the needs of the growing tissue, and, as in other homeostatic mechanisms, thus provide the stimulus for their increased production by the liver. Under such circumstances, according to Addis and his colleagues (9, 11), the nitrogen (protein) of the liver is increased.

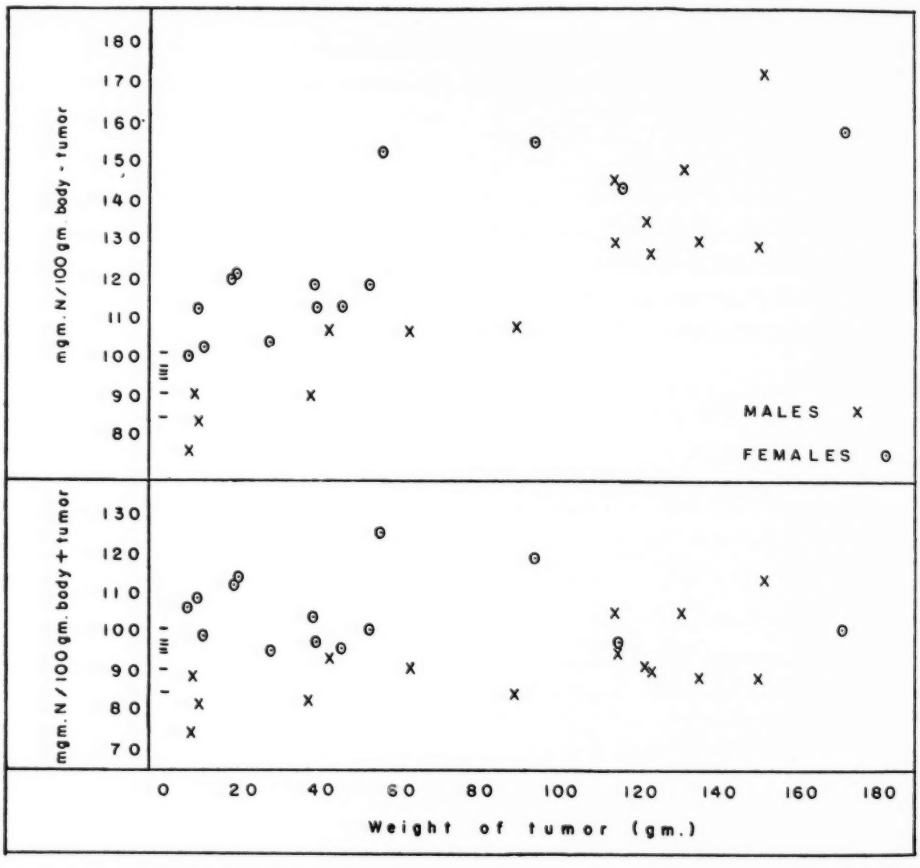

CHART 1.—The nitrogen content of the liver per 100 grams of body weight is plotted against the size of the tumor. In the upper portion of the graph, the nitrogen content is calculated per 100 grams of body weight minus the weight of the tumor, while in the lower portion the nitrogen content is based on the body weight plus the tumor weight.

SUMMARY

Twenty-two male Wistar albino rats, inoculated subcutaneously with methylcholanthrene, and fourteen females of the same strain, with spontaneous mammary tumors, were sacrificed at various stages of tumor size (0–171 gm.). The growths were encapsulated, and no metastases were seen. Body weight and tumor weight, wet and dry weight of the liver, and nitrogen content of the dry liver were determined. From these data the nitrogen of the liver, relative to body weight without the tumor and to body weight plus tumor weight, was calculated.

It was found that the nitrogen content of the liver increased proportionally with the total mass of the animal. In rats bearing large tumors, the relative amount of liver nitrogen was greater than expected when computed according to the weight of the carcass without the tumor; but, if calculated according to the weight of the carcass plus the weight of the tumor, the relative nitrogen content of the liver was the same as in normal rats.

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Influence of Pantothenic Acid Deficiency on the Viability and Growth of a Rat Fibrosarcoma*

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INTRODUCTION

It has been reported by Morris *et al.* (6, 7) that dietary pantothenic acid deficiency retarded the growth of spontaneous mammary tumors in mice (C3H strain). This result is in contrast to the findings of Bischoff *et al.* (2) who found no retardation of growth of Sarcoma 180 transplanted into Marsh-Buffalo mice on a similar pantothenic acid-deficient diet. Although different tumors and different strains of mice were used, the latter authors suggested that their negative results might be owing to failure to deplete the endogenous stores of pantothenic acid.

The present experiment was designed to test the effect on the growth of a rat fibrosarcoma of a diet deficient in pantothenic acid, as well as that of an anti-metabolite of this vitamin, ω-methylpantothenic acid (3).

EXPERIMENTAL

Thirty-six adult female rats of the Long-Evans strain were divided into four groups. Group I, containing ten control animals, was maintained on a modified Anderson and Smith (1) diet (Diet I, Table 1), and Group II, composed of nine control animals, was fed a modified Ershoff (5) diet (Diet II, Table 1). Groups III and IV, containing nine and eight experimental rats, respectively, were maintained on the modified Ershoff diet devoid of pantothenic acid. Group IV received, in addition to the deficient diet, injections of ω-methylpantothenic acid as described below.

After 1 week on their respective diets, the rats received inoculations of tumor tissue. A piece of fibrosarcoma tissue (4), approximating a volume of 30 c. mm., was implanted subcutaneously by trocar into each of two axillary and two inguinal ventral sites of each rat.

Just prior to i ıplantation of the tumor tissue, 200 mg. of ω-methylpantothenic acid (1 ml. of aqueous solution sterilized by passage through a Mandler filter) was injected subcutaneously into each rat in Group IV. The dose of the analog was reduced to 100 mg. on the second day and to 50 mg. on the third day after implantation. Injection at this dosage level was continued daily for the following 6 days. Since a rapid and continuous loss in body weight was observed, similar injections were given only on alternate days for the ensuing 2½ weeks. Normal saline was administered to the animals in Group III in an identical manner.

The growth of the tumors at each site was followed by estimating the maximum tumor area from caliper measurements. An initial increase followed by a 10 per cent or greater decrease in size or the total disappearance of the tumor was deemed regression.

TABLE 1

COMPOSITION OF THE DIETS

Diet I		Diet II	
Constituent	Per cent	Constituent	Per cent
Commercial casein	24	Vitamin-test casein*	22.0
Whole milk powder	24	Sucrose	73.2
Wheat germ+20 per cent middlings	20	Salt mixture†	4.5
Brewer's yeast	3	Corn oil supplement‡	0.75
Swiftning§	29	L-cystine	0.3
		Choline chloride‖	0.1

* Hot-alcohol extracted casein (General Biochemicals, Inc.) was employed. According to the manufacturer's specification, the maximum limit of pantothenic acid present was 0.45 μg. per gram. Nelson *et al.* (8) reported that this brand of casein contained 1.8 μg. of pantothenic acid per gram, primarily in the bound form.

† Salt mixture No. 1 of Sure (10).

‡ Constituents: Vitamin A, 20,000 USP units; Vitamin D, 2,000 USP units; α-tocopherol (Merck), 50 mg.; 2-methyl-1,4-naphthoquinone (Menadione), 1 mg.; corn oil (Mazola), 9.85 gm.

§ Swift & Co., Shortening made from animal and vegetable fats.

‖ Other vitamins added, in mg/kg of above diet: thiamin, 5; riboflavin, 10; pyridoxine HCl, 5; niacinamide, 100; inositol, 400; *p*-aminobenzoic acid 150; biotin, 0.2; and calcium pantothenate, 20.

* Paper 80. For the preceding related paper (paper 58) see Dunn *et al.* (4). This work was aided by grants from the American Cancer Society on recommendation by the Committee on Growth of the National Research Council, and the University of California. The authors are indebted to William Drell, who prepared the ω-methylpantothenic acid, and to Evelyn R. Feaver, Abraham M. Stein, and Edwin R. Skavinski, who participated in some of the preliminary studies.

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RESULTS AND DISCUSSION

It may be seen from the results given in Table 2 that there was no significant difference in the percentage of successful implantations (viability) of the tumor in the control and experimental groups. The number of established tumors which subsequently regressed, however, was greater in the experimental animals than in the controls. The percentage of tumors which regressed was not increased through use of the inhibitor.

An apparent retardation of tumor growth is indicated by the areas given in Table 2. The difference between the two control groups is relatively small, while that between the control and experimental groups is marked and roughly proportional to the degree of (dietary and induced) pantothenic acid deficiency. The similarity between the control groups is emphasized by the average terminal tumor weight data. While direct comparison between terminal tumor weights of the control and experimental groups is vitiated by the difference in time, the relatively low terminal tumor weight in Group IV substantiates the apparent retardation of tumor growth under this regimen evinced by the data on tumor area.

TABLE 2

BASIC DATA

Group	No. rats	No. sites inoculated	No. takes (per cent)	No. tumors regressed (per cent)	No. nonregressive tumors	Av. tumor size Days after implantation 17 (cm.²)	22 (cm.²)	27 (cm.²)	30 (cm.²)	34 (cm.²)	38 (cm.²)	Av. terminal tumor wt. (gm.)	Body weight data* Initial (gm.)	Final (gm.)	Diff. (gm.)
I. Anderson and Smith diet (control)	10	40	92	8.1	34	1.5	2.7	4.2				5.8	276	255	−21
II. Ershoff diet (control)	9	36	94	8.8	31	1.3	2.2	3.4	4.1			5.7	277	259	−18
III. Pantothenic acid-free diet (exper.)	9	36	92	27	24	0.18	0.78	1.8	2.4	3.5	5.1	7.7	253	220	−33
IV. Pantothenic acid-free diet plus inhibitor (exper.)†	8	32	100	28	23	0.25	0.92	1.3	1.8	2.5	3.2	4.3	263	194	−69

* Total weight minus tumor weight.

† ω-methylpantothenic acid.

While all the groups lost weight during the experiment, the only significantly different value was that for Group IV, in which the weight loss was approximately 3 times the average of the other three groups.

From the data in Table 3 it is apparent that the average rate of tumor growth, based on terminal tumor weights, was essentially the same for Groups I–III and significantly lower for Group IV. The reduced rate in this group is thought to reflect the markedly higher rate of body weight loss. This belief is supported by the similar ratios between average tumor weight and average body weight in Groups I–IV. An apparent inverse relationship between rate of tumor growth and rate of body weight loss is indicated by the curve given in Chart 1.

Schrek (9) has shown that the geometric mean of the diameters of a tumor (Walker rat tumor and Flexner-Jobling rat carcinoma) plotted against time yields a straight line, the slope of which represents the growth rate and whose intercept with the time-axis is a fair estimate of the "lag-period" of the growth of the tumor. The data of the present experiment have been treated in this manner (mean diameter = $\sqrt{d_1 \cdot d_2}$), and the resultant curves for nonregressive tumors are given in Charts 2 and 3. It may be seen in Chart 2 that the apparent retardation in growth rate shown by the areas tabulated in Table 2 results more from a difference in lag-period than from a marked difference in growth rate per se. The average latent period for the control groups was 2 days, while that of the experimental groups was 12 days. The superimposition of these curves (Chart 3), without regard for the time values, emphasizes the lack of significant differences in the growth rates for Groups I–III. The break in the curve for Group IV and the decreased slope of the second portion of the curve may reflect tumor-growth retardation. These results would lead to the conclusion that, with the possible exception of the inhibitor group (Group IV), there is no significant differ-

ence in the rates of tumor growth per se in the various groups but that there is definitely a greater "lag-period" in the initiation of active growth when a deficiency of pantothenic acid exists. The retardation in the rate of tumor growth in Group IV may be related, however, to the significant difference in body weight loss. Growth

TABLE 3

DERIVED TERMINAL DATA ON NONREGRESSIVE TUMORS

Growth	Av. rate tumor growth (gm/day)	Av. rate body weight loss (gm/day)	Av. tumor weight / Av. body weight*	Av. tumor weight (gm.) / Av. tumor size (cm.²)
I. Anderson and Smith (control)	0.21	0.78	0.023	1.4
II. Ershoff (control)	0.19	0.60	0.022	1.4
III. Pantothenic acid-free diet	0.20	0.87	0.035	1.5
IV. Pantothenic acid-free plus inhibitor†	0.11	1.8	0.022	1.3

* Average Standard Deviation = 0.017.
† ω-methylpantothenic acid.

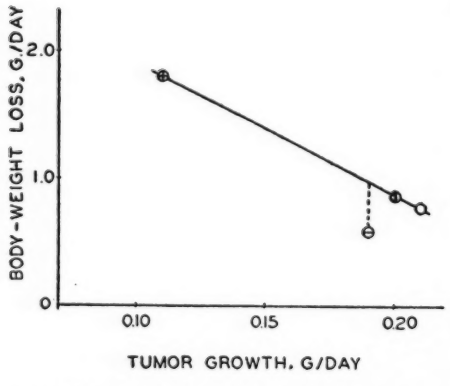

CHART 1.—Curve relating body weight loss and nonregressive tumor growth of female Long-Evans rats on various diets.

The notations are as follows:
○—Control Group I, modified Anderson and Smith diet.
⊖—Control Group II, modified Ershoff diet.
◑—Experimental Group III, Pantothenic acid-deficient diet.
⊕—Experimental Group IV, Pantothenic acid-deficient diet plus ω-methylpantothenic acid injection.

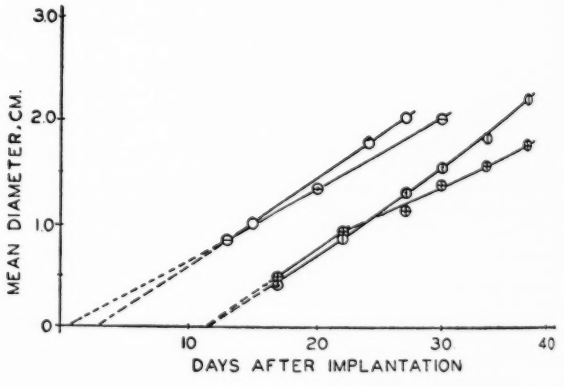

CHART 2.—Curves relating mean diameter of nonregressive tumors and time.

The notations are as follows:
○—Control Group I, modified Anderson and Smith diet.
⊖—Control Group II, modified Ershoff diet.
◑—Experimental Group III, pantothenic acid-free diet.
⊕—Experimental Group IV, pantothenic acid-free diet plus injections of ω-methylpantothenic acid.

curves for the regressive tumors are given in Chart 4.

The trocar method of implantation has been adopted as a standard procedure in this laboratory, since preliminary studies had shown that this method yielded more consistent results than the injection of a suspension of homogenized tissue. This result was ascribed to variations in the amount of tissue introduced by the latter method and to the possibility of infecting the tumor tissue. In a comparative study of the two methods, the deviation from the mean dry weight of injectable tissue in a series of seven uniform aliquots of a tumor suspension was 22.1 per cent, while the deviations for two sets of five trocar-implant samples were 5.0 and 9.6 per cent. Routine tests for bacterial infection of transplant material consisted in culturing sample pieces of tissue in fluid thioglycollate and in nutrient broth. Suspect cultures were then plated out on agar. Only once in five successive transplantations of trocar-implanted tissue was bacterial growth found, and it was not carried through by the tissue into subsequent transplantations. Infection of a pyogenic nature was encountered more often in the tissue inoculated in the form of a suspension and was eliminated, finally, by resorting to the trocar method. The tumor tissue implanted in the present experiment was shown to be bacteriologically sterile by the above tests.

Utilization of caliper measurements to follow the growth of tumors has been employed by many

authors. Schrek (9) has shown that the geometric mean of three diameters of a tumor, *in situ*, could be determined with an average error of 3.8 per cent, and that the average errors of the calculated tumor volumes and weights, based on the mean diameter, were within 10–11 per cent of the experimentally determined values. Voegtlin and Thompson (11) reported a uniform relationship between area (product of two diameters) and weight of transplants of Hepatoma 31. In the present experiment, the ratios of terminal tumor weight to terminal tumor size (area), given in Table 3, were all within 7.1 per cent of the mean value.

Ruffled hair, hyperirritability, exudate on the face and whiskers, blood-shot eyes, and other symptoms reported by Woolley (12) and others (3) as characteristic of pantothenic acid deficiency, were observed to a marked degree in Group IV and to some extent in Group III. That the retardation of the growth of the tumors may merely reflect the total systemic effect of pantothenic acid deficiency is indicated by the lack of significant differences between the ratios of terminal tumor weights to terminal body weights. This interpretation is in agreement with the conclusions of Morris *et al.* (7) who stated that ". . . retardation of tumor growth by a diet deficient in pantothenic acid results simultaneously in such severe interference in the host's nutrition that the procedure offers no practical application in adjunct tumor therapy."

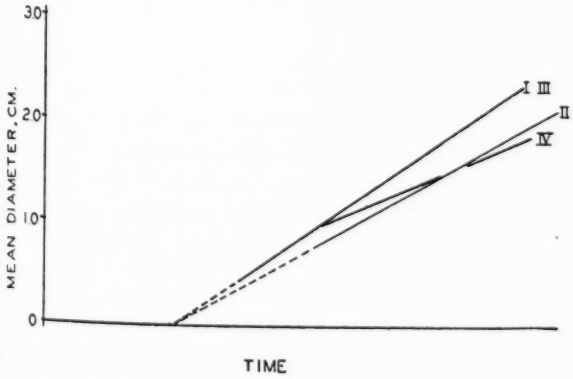

CHART 3.—Curves relating mean diameter of nonregressive tumors and time.

The Roman numerals refer to the group number.

The results of the present authors, as well as those of Morris *et al.* (6, 7), differ from those of Bischoff *et al.* (2). It is interesting to note that Morris *et al.* and Bischoff *et al.* also obtained diametrically opposite results with pyridoxine deficiencies. This lack of similarity might be ascribed, tentatively, to differences in the vitamin requirements of the types or strains of animals or tumors employed.

CONCLUSIONS

It is concluded from these experiments that pantothenic acid deficiency for 1 week prior to inoculation of a fibrosarcoma in rats had no effect upon the viability of the tumor implants. Continued deficiency caused an apparent retardation of growth of the tumors and increased the percentage of regressions. The retardation of growth was shown to derive from an increased "latent" period rather than from a decreased growth rate per se. The injection of a pantothenic acid antimetabolite, ω-methylpantothenic acid, into animals on a pantothenic acid-deficient diet did not further increase the percentage of regression or the length of the "latent" period. The decreased tumor growth of the animals treated with the inhibitor apparently relates directly to the significantly greater body weight loss under this regimen. The retardation of tumor growth by pantothenic acid deficiency appears merely to reflect the total systemic effect of such a deficiency and does not indicate a specific effect on tumor tissue.

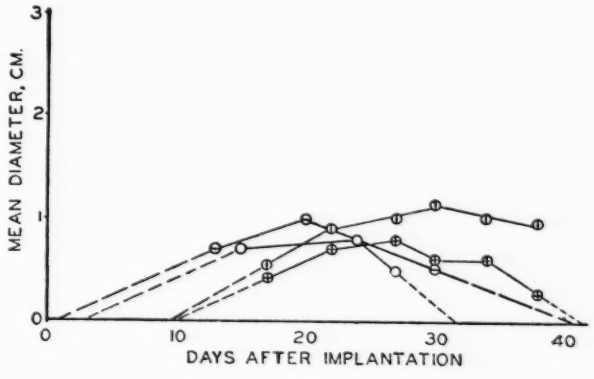

CHART 4.—Curves relating mean diameter of regressive tumors and time.

The notations are as follows:

○—Control Group I, modified Anderson and Smith diet.

⊖—Control Group II, modified Ershoff diet.

◑—Experimental Group III, pantothenic acid-free diet.

⊕—Experimental Group IV, pantothenic acid-free diet plus ω-methylpantothenic acid injection.

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Recovery of Crown-Gall Tumor Cells

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Crown-gall tumor cells isolated from many plant species, including *Helianthus annuus* L. (5, 19) and *Vinca rosea* L. (3, 18), have been found to be permanently altered cells that reproduce true to type and against the growth of which there is no adequate control mechanism in the host. These characteristics are generally considered to be the essential criteria by which malignant animal cells are distinguished from healthy or inflammatory cells. In emphasizing the permanency of the cellular alteration, Kidd (6) states that "Cancer cells remain such until they die . . . they never turn back en masse towards the benign or normal state. . . ." The inability to accomplish a reversal of malignant to normal cells under controlled conditions has, however, served as a serious barrier to further progress in defining more precisely the nature of the proximate cause for the continued abnormal proliferation of certain neoplastic cells.

The initiating and continuing factor or factors responsible for the alteration of normal cells to tumor cells and for the continued abnormal proliferation of the tumor cells once the cellular change has been consummated remain uncharacterized in crown gall. The former are clearly associated with the presence of the inciting bacterium during the inception period of the disease. The author has shown (1, 4) that this factor is capable of bringing about the cellular alteration as early as 34–36 hours after the bacteria are brought into contact with susceptible tissues. When the inciting bacteria are destroyed by thermal treatment at this early period, very small, extremely slowly growing galls are produced. If, on the other hand, the bacteria are permitted to act on the host cells for 4 days before being killed, rapidly growing tumors result. Such tumors are not self-limiting, and when they develop on certain perennial plants they may reach enormous size. The transformation of normal cells into tumor cells appears to take place gradually, leading in a 4-day period to a rapidly growing autonomous type of cell.

Essentially two hypotheses have been advanced to account for the continued abnormal proliferation of these tumor cells. It has been suggested (2) that crown-gall tumor cells have acquired, as a result of their transformation, a capacity for producing, in greater than regulatory amounts, a growth-promoting substance. The continued and unregulated production of such a substance could and probably would account for the continued abnormal proliferation of the tumor cells. That large amounts of some growth-promoting substance are being generated by crown-gall tumor cells is obvious from several different types of evidence (2, 13). However, it is difficult to determine from available information whether the observed growth-substance imbalance following the cellular alteration is the cause or the result of the rapid autonomous growth of the altered cells.

A second hypothesis advanced to account for the continued abnormal proliferation of crown-gall tumor cells postulates the presence in those cells of self-duplicating cytoplasmic entities (13). It is well known that such cytoplasmic factors can sometimes be eliminated from cells under conditions that favor the more rapid multiplication of those cells in relation to the multiplication of the self-duplicating factor. Sonneborn (17) indicates, for example, that the cellular concentration of the cytoplasmic factor, kappa, is determined in part by external conditions. By increasing the quantity of available food, the cells of the Paramecium can be made to multiply at a faster rate than does the cytoplasmic particle. As a result, the concentration of kappa per cell is reduced, and the organism may free itself entirely of that entity if the culture is maintained under conditions that favor rapid multiplication of the Paramecium. Kunkel (7) has obtained similar results in his studies on the mosaic of sugar cane, which is a virus disease.

It seemed possible, therefore, to test the second hypothesis if conditions could be established that would permit an unusually rapid division of the tumor cells.

Normal meristematic plant cells in a rapidly growing bud divide with far greater frequency than do most crown-gall tumor cells, despite the fact that tumors increase greatly in volume. It

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was therefore hypothesized that, if crown-gall tumor cells could be made to organize buds and if those tumor buds could be forced into rapid growth, recovery of the tumor cells might be accomplished provided the factor responsible for the continued abnormal cellular proliferation in crown gall was subject to the effects of dilution in very rapidly dividing cells. It is with this problem that the present paper is concerned.

METHODS

Turkish tobacco (*Nicotiana tabacum* L.) was used as the test plant in the experiments. The plants were grown in 6-inch pots in a greenhouse. When they reached a height of 8–12 inches, a single cut with a sharp razor was made through an internode close to the top of the plant. Bacterial inoculations of the freshly cut surfaces were made with the T37 strain of the crown-gall organism. To prevent drying, the inoculated area was covered for several days with a rubber tape known as "Sterilastic." In order to induce a maximum stimulus for regeneration in the host, all normally produced axillary buds were removed from the inoculated tobacco plants.

White's medium (19) containing 0.1 per cent agar was used for culturing normal and tumor cells. The tissues were grown in 125-cc. Erlenmeyer flasks to which about 30 cc. of the medium was added. All cultures were permitted to develop in diffuse light. The crown-gall bacterium grows well on White's medium, and if plant tissues are contaminated with this organism they are quickly overgrown by the bacterium. As an added precaution, however, tumor tissues of the various clones were macerated periodically and plated on Difco beef extract peptone dextrose agar to determine the bacteriological sterility of these tissues. The grafting technic employed was similar to that described earlier (19).

EXPERIMENTAL RESULTS

To test experimentally the working hypothesis set forth in the introduction of this paper, it was necessary to isolate crown-gall tumor cells that possessed a capacity for organizing buds. Crown-gall tumor cells isolated from many plant species and cultured *in vitro* have been characterized by their rapid and autonomous powers of proliferation and their limited capacities for differentiation and organization. About 35 years ago, however, Magnus (12) and Smith (14) independently described, as occurring on certain plant species, highly complex crown-gall tumors that were composed of unco-ordinated tumor cells and of tissues and organs that showed a high degree of structural organization. Similar teratomata have since been found to develop on certain other plant species following inoculation with the crown-gall bacterium (3, 8, 9–11, 14–16). It has not been determined, however, whether the shoots and stemlike structures that develop from the teratomata are composed of normal cells that have been stimulated to grow by the developing tumor, or whether they are composed of tumor cells that have acquired a capacity to organize.

Complex tumors similar to those described earlier were produced on tobacco plants by inoculating the cut stem surface with the inciting bacteria. Following inoculation, crown-gall tumors developed in the region of the cambium. As the new growth expanded, areas of more or less well organized tissues developed from them. The overgrowths grew rapidly into complex tumors similar to that shown in Figure 1. These teratomata were characterized in part by the very grotesque morphological picture that they presented. Many of the shoots and stemlike structures that developed from them were highly distorted.

To determine whether the abnormally organnized structures found to develop from the complex tumors were composed of tumor cells, fragments of tissue were isolated from the structures and planted on White's tissue culture medium. Normal cells isolated from tobacco stems grow very slowly on this medium (Fig. 5) and increase in volume only slightly even after prolonged incubation. Bacteria-free crown-gall tumor cells, on the other hand, grow profusely and in an unco-ordinated manner on this medium, as shown in Figure 7.

It was possible to demonstrate with the use of this differential medium that the morphologically distorted but organized stemlike structures that developed from the complex tumors were composed of cells whose metabolism was fundamentally different from that found in normal tobacco cells isolated from comparable but normal stem segments. Bacteria-free fragments of tissue isolated aseptically from abnormal stemlike structures grew profusely on the medium (Fig. 6), as did previously cultured bacteria-free crown-gall tumor tissue. These cells differed from tobacco tumor cells of the type studied in the past, however, in that they indefinitely retained a capacity for organization. The surfaces of the cultures were covered with small adventitious buds and leaves. Some appeared fairly normal, while others were greatly distorted. The buds and leaves, when attached to the masses of tissue, did not grow appreciably in culture, reaching a maximum length of only a few millimeters.

One clone of this tissue has been maintained in culture for about 20 months. Three clones, isolated from other sources, have been cultured for somewhat more than 1 year. These four tissues have been studied in detail. In all instances the cells have maintained without alteration their characteristic properties of growing profusely on a culture medium that does not support continued growth of normal cells and of retaining indefinitely the capacity for organizing buds and leaves.

Numerous attempts to isolate small fragments of tissue from the four clones that would develop in culture in an essentially undifferentiated and unorganized manner, as did the tumor tissue previously described, have thus far failed. Although these fragments were selected because of their essentially undifferentiated growth at the time of isolation, they were found to possess during their development the same capacity for organizing leaves and buds as did the tissues from which they were originally derived.

Several hundred small but well organized leaves that developed from the abnormal tissue masses in culture were studied. The leaves, which ranged in length from 2 to 3 mm., were white or light green in color. They were removed aseptically and cut into two or three pieces. Each piece was planted on White's medium and permitted to develop. Some of the fragments turned brown and died soon after isolation. Most, however, grew profusely, again producing gall tissue masses more or less covered with buds and leaves in a manner similar to that found in tissues from which the organized leaves were originally isolated. Since histological studies demonstrated that there were no preformed buds or leaf anlagen in the tissue fragments at the time of isolation, the results indicated that the cells of the very small but morphologically well organized leaves possessed the same potentialities for profuse growth in culture as did the gall tissues from which those leaves were derived.

When small fragments of these bacteria-free tissues, some of which had been carried in culture for more than 1 year, were implanted at the cambial level into an internode of healthy tobacco plants, they commonly developed into overgrowths that reached a diameter of somewhat more than 1 cm. in a 6–8-week period (Fig. 4). These tumors developed more slowly, and they did not show the same degree of organization that was found when similar tissue fragments were grafted onto the tips of the plants from which all normal axillary buds were removed. The latter developed rapidly into large overgrowths that were for the most part organized into highly distorted leaves and stems, as shown in Figure 3.

The capacity of these cells to grow rapidly and indefinitely on a culture medium free of added growth substances and the ability of the cells to develop independently of the growth-restraining influences of the host are characteristic attributes of tumor cells. These tumor cells, however, differ from the unco-ordinated crown-gall tumor cells of the type isolated and studied in the past in that they retain indefinitely *in vitro* and *in situ* a well developed capacity for organizing buds and leaves.

Recovery of tumor cells.—The finding that the morphologically abnormal but organized stemlike structures that developed from the complex tumors were composed of tumor cells made it possible to determine whether complete recovery of those tumor cells could be accomplished when the tumor buds were forced into very rapid growth. Morphologically abnormal shoots, some of which were similar in appearance to the one shown in Figure 1*a*, were removed and grafted to healthy tobacco plants. One such graft, which is representative of the type used in this study, is shown in Figure 2. The picture was taken somewhat more than 2 months after the abnormal shoot had been grafted onto the healthy stock. During this period the scion increased slowly and very abnormally in length as well as in thickness. The expanding cells ruptured the epidermis at many points along the stem, resulting in new growths that were composed of disorganized cells and adventitious buds. Many fragments of tissue were isolated from various parts of the abnormal shoot and planted on the culture medium. All were found to grow as tumor cells that had retained a well developed organizational capacity, thus indicating that the entire structure shown in Figure 2 was an organized tumor. Axillary and adventitious buds developed at the apex of the shoot (Fig. 2*a*). When these buds produced shoots large enough to be used as scions, the shoots were removed and grafted to tobacco plants from which all normal buds had been removed. The new growth that resulted from these rapidly growing shoots gradually became more and more normal in appearance. The tips of the shoots were again removed and grafted to healthy plants. They developed rapidly and appeared normal in every respect, ultimately flowering and setting seed. Fragments of tissue isolated from normal-appearing stems derived from the tumor tissue buds grew as poorly in culture as did healthy tobacco tissue. Recovery appeared to have been complete. It was a gradual process that progressed in the direction of the normal as affected shoots

developed and were forced into rapid growth. These experiments were repeated with similar results when bacteria-free fragments of the tumor tissues of the type that possessed the capacity to organize were grafted to the cut stem end of a tobacco plant from which all normally formed axillary buds were removed. The tumor cells had been maintained in culture for more than 1 year at the time when the grafts were made. Recovery of bacteria-free tumor buds has also been accomplished *in vitro* by forcing certain of the more normal-appearing buds isolated from the tumor mass into rapid growth on a culture medium.

Histological studies.—A histological study of possible changes that occur during the process of recovery was undertaken. Representative shoots and stemlike structures that ranged in appearance from morphologically highly distorted to well organized shoots were studied in detail. Pieces of the desired tissue were removed from these structures and placed in formalin-acetic acid-alcohol fixative, dehydrated, imbedded in paraffin, sectioned, and stained. Adjoining fragments isolated under aseptic conditions from immediately above and below the point at which the fixed tissue had been removed were planted on White's medium. In this way it was possible to determine whether recovery had occurred in any of the organized structures.

Small fragments of tissue isolated from the more or less morphologically abnormal structures grew profusely on the culture medium. A transverse section through one such fragment following a 3-week incubation period on the culture medium is shown in Figure 13. The new growth was composed for the most part of rapidly proliferating disorganized hyperplastic cells, together with small centers of organization. Figures 14 and 15 picture sections of the new growths that had been removed from the original fragments, planted on fresh agar, and permitted to develop for about 1 month. These tumor tissues were composed mostly of hypertrophied and hyperplastic parenchymatous cells. Some organized leaves that were characteristically found arising from these tissues are shown in cross-section in Figures 14 and 15. Although morphologically these structures were leaves, they did not show the well differentiated cell types that are normally associated with tobacco leaves. Many leaves were direct outgrowths of the tumor tissues and were composed of the same parenchymatous cell types that were found in the disorganized cell masses from which they arose.

A total of 226 fragments, isolated from 31 morphologically organized structures that originated from 9 teratomata, were planted on the culture medium. Of the tissue fragments isolated, 3 were contaminated by a white slimy bacterium, while molds developed in 39 flasks. These 42 flasks were discarded, and the remaining 184 were retained for study.

The results of these experiments showed that all fragments isolated from morphologically more or less abnormal structures developed profusely on the culture medium. With few exceptions they possessed a well developed capacity for organization. Twelve fragments isolated from two well organized though slightly thickened stems grew slowly in culture in a mostly undifferentiated and unorganized manner. The cells of these fragments lacked the organizational capacity shown by cells isolated from other teratological structures. Thirty-four fragments isolated from six normal-appearing shoots that developed from the bacterial-incited teratomata grew very poorly in culture, indicating that they had recovered completely very early in their development from the effects of the tumor-inducing principle.

Fragments of tissue isolated aseptically from highly abnormal stems, such as the one shown in Figure 1*b*, grew rapidly on the culture medium and showed a well developed organizational capacity. Despite the very abnormal appearance of these structures, histological studies demonstrated (Fig. 9) that a definite attempt had been made by the cells to differentiate and organize. There is, in part, a well defined epidermis and cortex, a great increase in secondary xylem, and a highly abnormal pith.

Shoots similar to the one shown in Figure 1*a* were not only morphologically well organized, but sections prepared from a point along the stem, indicated by the arrow in Figure 1*a*, showed that histologically the cells of the stem were also well differentiated and organized, as demonstrated in Figure 10. Epidermis, cortex, internal phloem, and pith were well developed. The vascular system was well defined, although there was a considerable increase in secondary xylem. Fourteen fragments of tissue isolated from different parts of the shoot grew readily in culture, indicating that the entire structure was an organized tumor. The results demonstrate, therefore, that under certain conditions crown-gall tumor cells are capable of a very high degree of differentiation and organization.

Some of the shoots that developed from the teratomata were morphologically quite distorted at their base, when they had attained a length of several inches, while the apical portions of the structures were normal in appearance. Tissue isolated from four different shoots of that kind grew profusely on the culture medium when obtained from abnormal regions of the base. They developed poorly, as does normal tissue, when isolated from

the apical region. A histological section prepared from one of the recovered shoots is shown in Figure 12. This tissue appears to be normal in every respect. For comparison, a cross-section of a normal adventitious stem is presented in Figure 11.

In view of the fact that shoots of plants increase in size as a result of the rapid division and subsequent elongation of the cells of an apical bud, the results suggest that recovery of cells from the effects of the tumor-inducing principle is associated with the rapid division of the meristematic cells at the apex of the shoot.

Influence of polarity on tumor development.—During the course of these studies it was observed that, when cells of a cut stem surface of tobacco plants were inoculated with crown-gall bacteria, a complex overgrowth or teratoma resulted. When, on the other hand, this same organism was introduced into internodes of a stem containing an apical bud, typical undifferentiated crown-gall tumors developed at the points of inoculation. It appeared likely, therefore, that the type of tumor initiated was a function of the regenerative competency and relative potency of the host cells acted upon by the tumor-inducing principle at the time of cellular alteration. This was tested experimentally by studying the effects of polarity on the response of the host to inoculation with crown-gall bacteria. Normal tobacco stem cuttings commonly show, under suitable conditions, a pronounced polarity in which shoots are formed at the apical end of a cutting and roots are produced at the basal end.

Tobacco plants 8–10 inches tall were cut through an internode at about the middle of the plant. The apical bud and other leaves were permitted to remain on the upper half of the plant. The lower portion in some instances contained the original root system, while in others the stems were severed at ground level. The original leaves were kept on the lower stem segments. In all instances the cut ends of the cuttings were immersed in moist sand and covered with bell jars. After 3 weeks to a month the cuttings were transplanted to compost soil in 8-inch pots and kept on a greenhouse bench.

With the exception of the untreated controls, the two stem surfaces of the original cut were inoculated with crown-gall bacteria. The cells of these stem surfaces prior to the time of their separation possessed the same potentialities, since they were adjoining cells of the same stem. Immediately after the cut was made, however, the cells below the point at which the razor had severed the stem became the apical cells of the basal portion of the original plant, while cells above the cut became the basal cells of the upper cutting. The stimulus for bud formation was pronounced in the cells at the apical end, while the cells at the basal end of the shoot possessed the stimulus for forming buds to a far smaller degree or not at all. The potentialities of the cells at the two surfaces were therefore very different after the cut had been made.

Undifferentiated and unorganized crown-gall tumors were always produced at the basal end of the upper portion of the original plant, as shown in Figure 8*a*. The other inoculated surface that had become the tip of the lower half of the plant, following the cut, produced typical teratomata (Fig. 8*b*). The results demonstrate, therefore, that the position of a cell at the time of its alteration is a decisive factor in determining the type of tumor that will develop as a result of the action of the tumor-inducing principle. Further studies on the influence of polarity on tumor development will be taken up in detail elsewhere.

DISCUSSION

It is well known (7, 17) that certain self-duplicating cytoplasmic particles can be eliminated from cells under conditions that favor the more rapid multiplication of those cells in relation to the increase in numbers of the cytoplasmic entity. Such factors have been postulated (13) as being responsible for the continued abnormal proliferation of crown-gall tumor cells. It was therefore assumed, as a working hypothesis, that, if crown-gall tumor cells could be made to divide with sufficient rapidity, a dilution and final elimination of the tumefacient factor might be accomplished with the resulting recovery of the tumor cells.

A very rapid division of plant cells can be effectively encouraged by forcing the growth of a bud. The cells of such meristematic tissue divide with far greater frequency than do most crown-gall tumor cells. By permitting the tumor-inducing principle associated with the inciting bacteria of this disease to alter pluripotent cells that possessed at the time of their alteration highly developed regenerative powers, tumor cells were obtained that retained indefinitely a well developed capacity for organizing buds. Shoots derived from such adventitious tumor buds were forced into very rapid growth by a series of graftings to healthy plants. When these tumor cells were thus made to divide with great rapidity, they gradually recovered and became normal in every respect. This finding suggests that the factor which causes crown-gall tumor cells to develop abnormally becomes diluted in and is eventually lost from tumor cells that are forced to grow and divide with sufficient rapidity.

The relative potency and regenerative compe-

tency of the host cells acted upon by the tumor-inducing principle at the time of their alteration appear to be important if not decisive factors in determining the type of tumor that will result. The cytological system through which the potentialities of a cell manifest themselves is, of course, a matter of conjecture. Evidence presented here suggests, however, that polarity may play an important part.

When host cells that have a low potency at the time of their alteration are transformed by the bacteria, the resulting tumor cells are characterized by their rapid and autonomous powers of proliferation and their very limited powers of differentiation and organization. When, on the other hand, plant cells that possess great developmental potentialities at the time of alteration are acted upon by the bacteria, those tumor cells may retain indefinitely a well developed capacity for organizing buds. This organizational ability is an expression of the inherent potentialities of the tumor cells, and it does not in itself appear to affect directly the recovery of those cells. It is only when the cells of a tumor bud are made to divide very rapidly that recovery of the affected cells is accomplished.

A basic difference in the regenerative capacity of cells of higher animals and plants does, of course, exist. The character of somatic cells of higher animals is often specifically determined and strongly fixed very early in their development, while somatic cells of certain higher plants may and frequently do preserve a high degree of pluripotency. A single plant cell, or a small group, under the proper stimulus is capable of regenerating an entire plant. Plant cells behave in this respect as do certain cells of lower animals which may also possess well developed regenerative powers. These facts should be kept clearly in mind in any discussion of the findings concerning recovery of crown-gall tumor cells.

SUMMARY

Under the proper stimulus the cells of many plant species, including tobacco, possess highly developed regenerative powers. When such pluripotent cells are transformed by the tumor-inducing principle associated with the crown-gall bacterium, the resulting tumor cells may retain indefinitely a well developed capacity for organizing buds. Adventitious shoots derived from such tumor buds were forced into fast growth by a series of graftings to healthy plants. When these tumor cells were thus made to grow and divide very rapidly, they recovered and became normal in every respect. This finding suggests that the factor which causes crown-gall tumor cells to develop abnormally becomes diluted in and is eventually lost from affected cells that are forced to grow and divide with sufficient rapidity.

The regenerative competency of the host cells acted upon by the tumor-inducing principle at the time of their alteration appears to be decisive in determining the type of tumor that will result. Although the cytological system through which the potentialities of a cell manifest themselves is not known, the results reported here indicate that polarity plays an important part.

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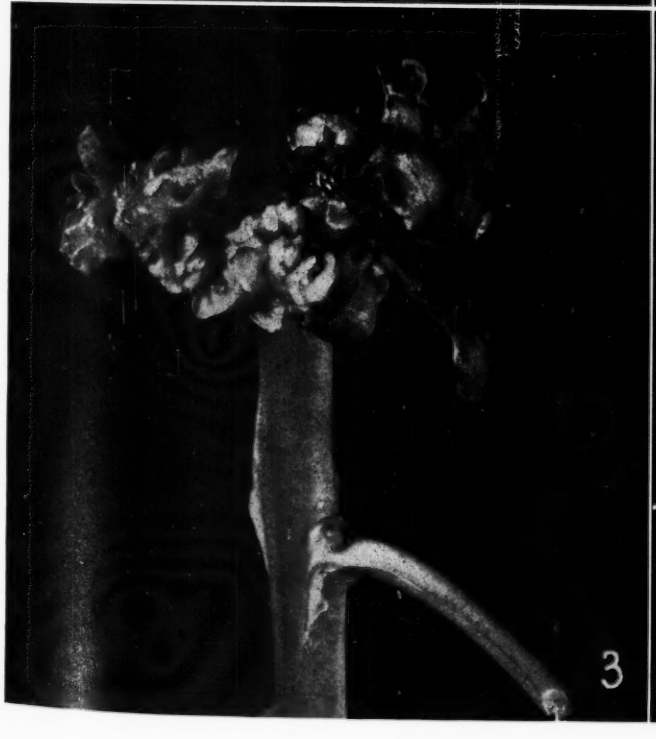
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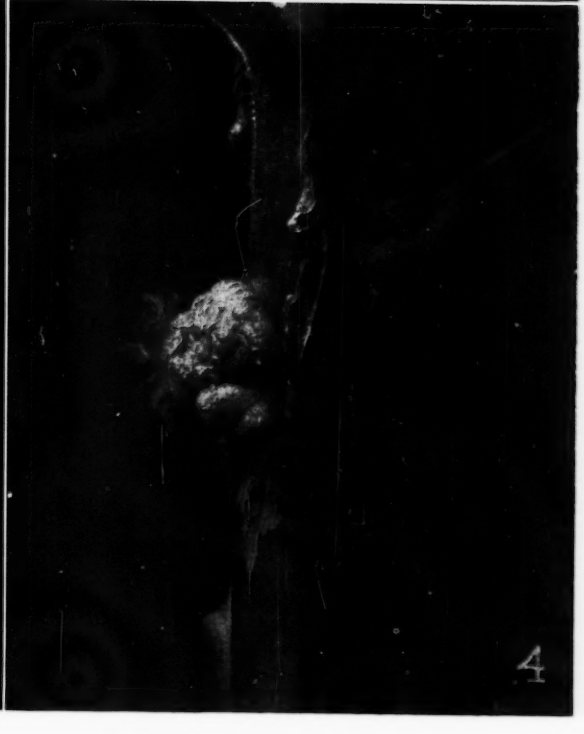
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FIG. 1.—Teratoma incited by bacteria. The cut stem end of a tobacco plant, from which all axillary buds had been removed, was inoculated with the T37 strain of the crown-gall bacterium.

FIG. 2.—Morphologically abnormal shoot grafted to the tip of a normal tobacco plant. At the time of grafting, the shoot, which was shown to be an organized tumor, was similar in appearance to the shoot shown in Figure 1 *a*. The picture was taken somewhat more than 2 months after the graft was made.

FIG. 3.—Bacteria-free teratoma. A fragment of tissue obtained from a culture, similar to that shown in Figure 6, was grafted to the cut stem end of a tobacco plant from which all axillary buds had been removed.

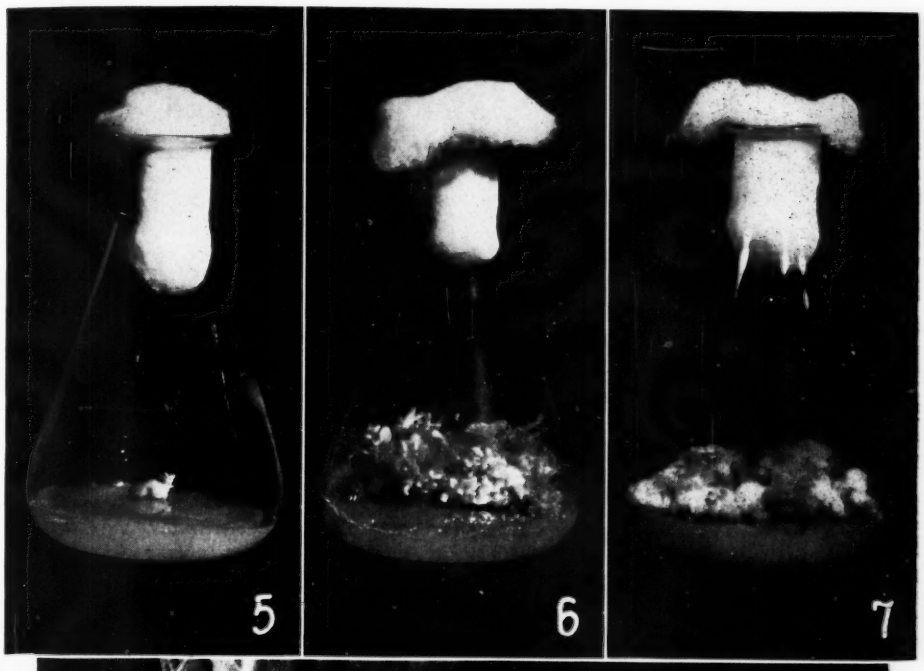
FIG. 4.—Bacteria-free fragment of tumor tissue, similar to that used in the experiment shown in Figure 3, was implanted at the cambial level into an internode of a tobacco plant that contained an apical bud.

FIG. 5.—A tissue culture of normal tobacco stem tissue. Such cultures develop very slowly on White's medium, and their growth is limited.

FIG. 6.—A bacteria-free culture originally isolated from an organized tumor similar to that shown in Figure 2. These tumor cells grow profusely on White's medium and retain indefinitely a capacity to organize buds and leaves.

FIG. 7.—A bacteria-free culture of crown-gall tumor tissue originally isolated from an undifferentiated and unorganized tumor similar to that shown in Figure 8 *a*. These cells are characterized by their rapid and autonomous powers of proliferation and their very limited powers of differentiation and organization.

FIG. 8.—Results showing the influence of polarity on tumor development. The cuttings *a* and *b* were derived from a single tobacco plant. The plant was cut through an internode, and the freshly cut surfaces were inoculated with the bacteria. A typical undifferentiated and unorganized tumor developed at the basal surface of what was originally the upper half of the plant, as shown in Figure 8 *a*. A teratoma was produced at the apical surface of the lower half of the original plant.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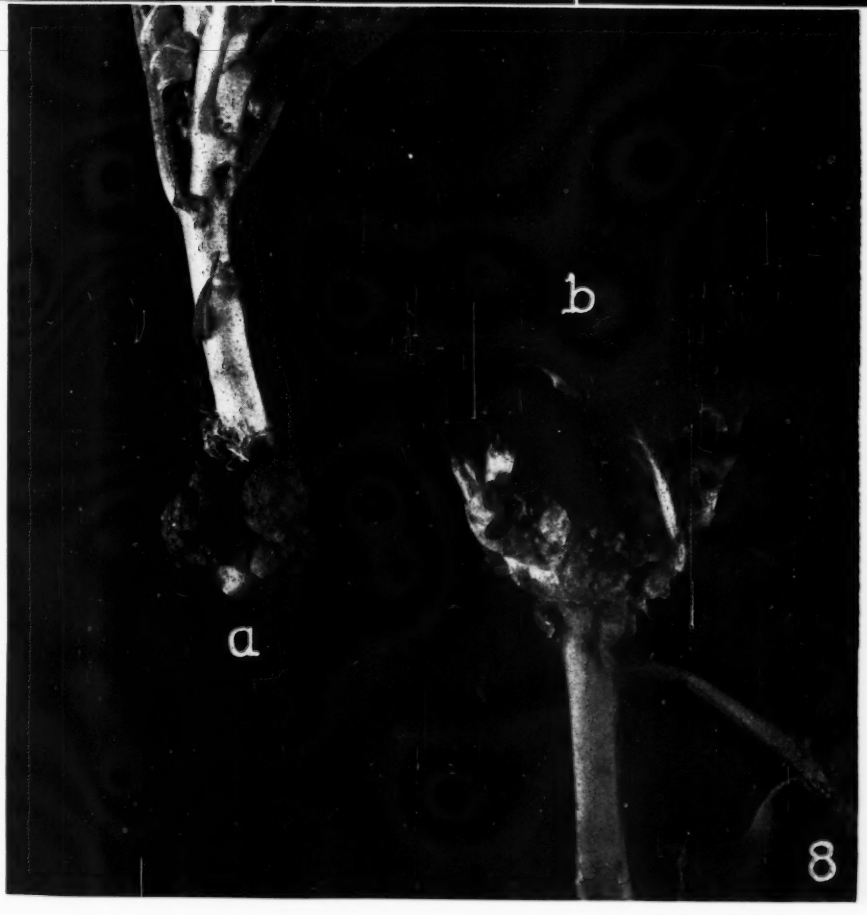
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FIG. 9.—Transverse section of the morphologically very abnormal stemlike structure shown in Figure 1 *b*. There is, in part, a well defined epidermis and cortex, a great increase in secondary xylem, and a highly abnormal pith.

FIG. 10.—Transverse section of the abnormal shoot shown in Figure 1*a*. The tissue was taken from that part of the shoot indicated by the arrow. Despite the high degree of differentiation and organization of the cells, the shoot was found to be an organized tumor.

FIG. 11.—Transverse section of a normal adventitious shoot of a tobacco plant.

FIG. 12.—Transverse section of a fully recovered shoot that was derived from a tumor tissue bud.

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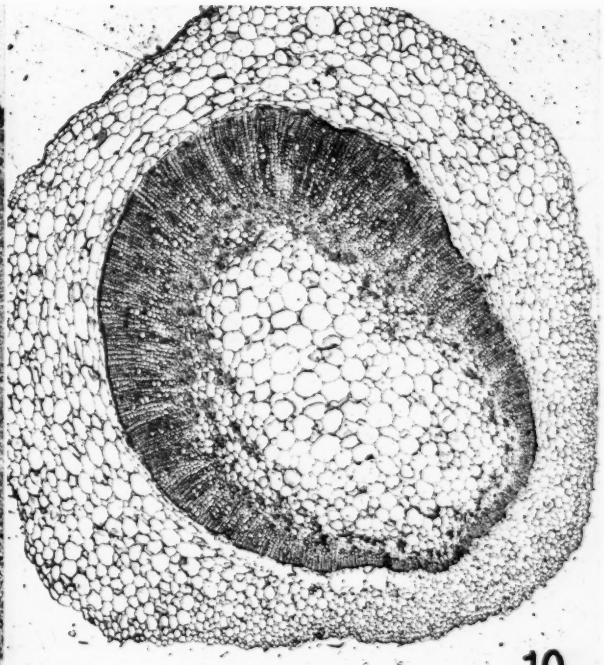

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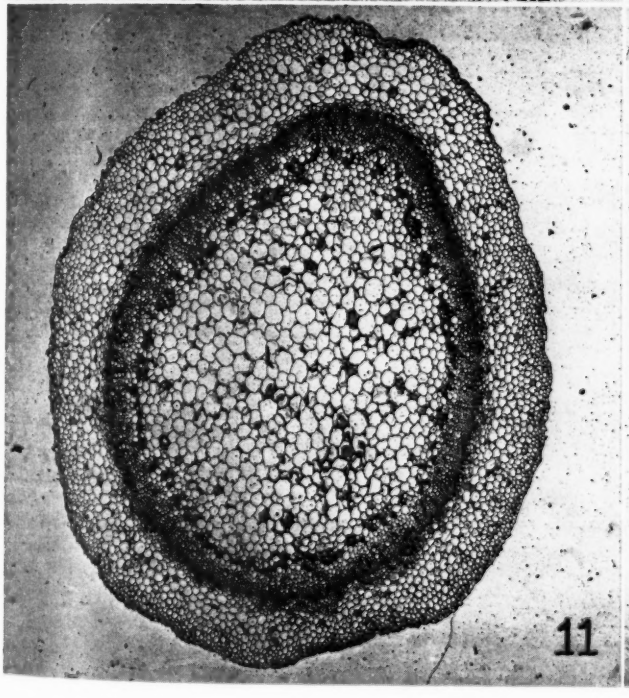

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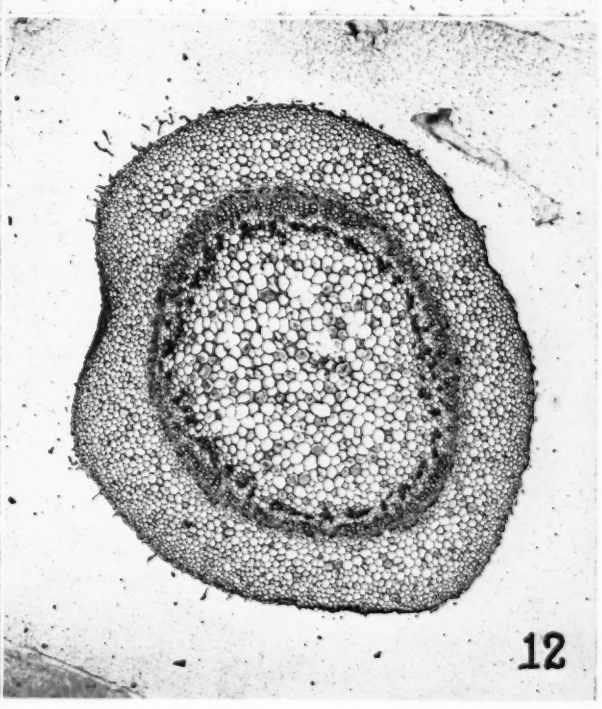

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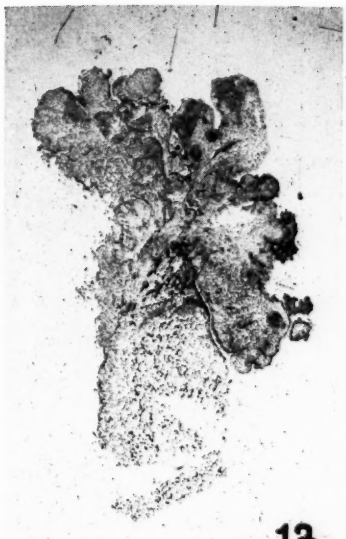
FIG. 13.—Transverse section of tissue isolated from a teratological stem. The tissue was sectioned following a 3-week growth period of the tissue fragment on White's medium.

FIGS. 14 and 15.—Transverse sections of bacteria-free teratoma that had developed on White's media for about 1 month. The leaves are indicated by the arrows.



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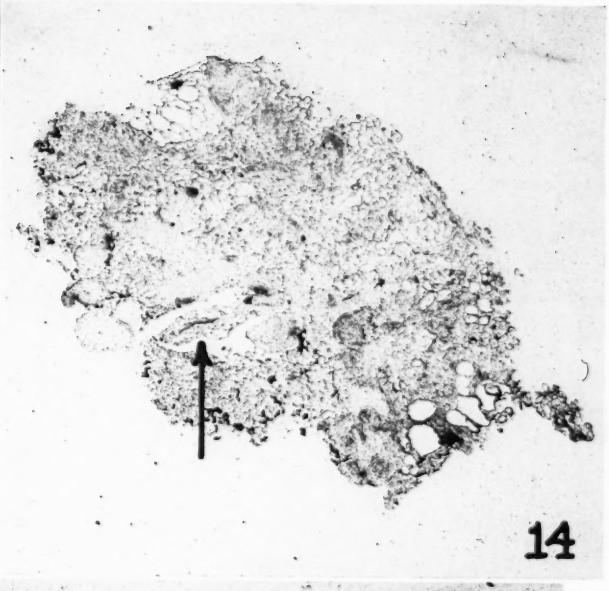

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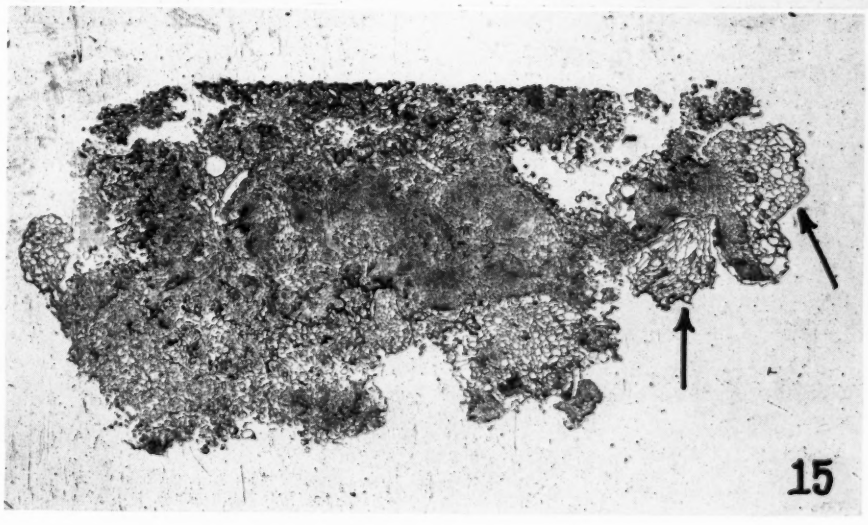

15

Metabolism of Neoplastic Tissue

I. The Oxidation of Carbohydrate and Fatty Acids in Transplanted Tumors*

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As part of a program of study of the intermediary metabolism of neoplastic tissues the present investigation was undertaken to obtain information concerning the carbon sources and the pathways of respiration in a series of transplanted tumors of the mouse and rat. Ever since the disclosure, by Warburg, of the high aerobic glycolysis of tumors (21), the general conception has prevailed that neoplastic tissue is characterized by a disturbance or inadequacy of oxidative metabolism. This view has been extended and elaborated by a number of investigations which indicated that (*a*) oxidation of carbohydrate intermediates in minced or homogenized tumors was considerably lower than in comparable preparations of normal tissues (5, 15, 16) and (*b*) tumors were generally low in factors involved in electron transport (7, 12, 13, 17, 19). On the other hand, it was already evident from Warburg's experiments (21) that oxygen consumption by the relatively intact cells of tumor slices *in vitro* is similar in magnitude to that of normal tissue slices, a well substantiated property of tumor cells which is incompatible with defective respiratory mechanisms.

It appeared that the question of what substances are utilized for oxidative metabolism in tumors could be directly approached by studying the oxidation of carbon-14-labeled substrates by neoplastic tissue slices *in vitro*. Since surviving slices of tumors carry on an energetic respiration, it was anticipated that the appearance of labeled carbon in the respiratory carbon dioxide would afford straightforward proof that the substrate in question was oxidized; and the magnitude of C^{14} incorporation should provide a reliable measurement for comparison of oxidative capacity of tumors with that of normal tissues.

In the present study the oxidation of C^{14}-labeled glucose, carboxyl-labeled lactic acid, and carboxyl-labeled palmitic acid by a number of transplanted tumors of the mouse and rat was compared to that of a representative group of normal tissues. The results demonstrated the capacity of neoplastic tissues to oxidize all three substances about as rapidly as do normal tissue slices. Evidence for the participation of the citric acid cycle in the oxidation of C^{14}-labeled glucose and palmitic acid was obtained by inhibition experiments with *trans*-aconitate, and more definitely by isolating radioactive citric acid as an intermediary product.

METHODS

Labeled compounds.—Carboxyl-labeled lactic acid and glucose labeled equally in all positions were obtained from the Isotopes Division of the U.S. Atomic Energy Commission; the carboxyl-labeled palmitic acid was prepared as described in a previous communication (23). Radioactivity measurements were made with a thin window or a gas-flow counter. Unless otherwise specified, samples were counted as barium carbonate in "infinitely thick" layers of 7.5 sq. cm. area. Activities are given as counts per minute per dish.

Tumors.—The neoplastic tissues used in this investigation were chosen because they could be grown as subcutaneous transplants to rather large size without undue necrosis, and gave firm, resilient tissue which could be sliced easily in quan-

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† Part of a dissertation to be presented by Mr. Charles E. Wenner in partial fulfillment of the requirements for the Ph.D. degree in the Graduate School of Temple University. A preliminary report of part of this work has appeared (J. Am. Chem. Soc., **72**:4332, 1950).

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tity. The rat hepatoma was obtained through the kindness of Dr. W. F. Dunning (4), and the mouse hepatoma was obtained from Dr. Julius White (6). The other tumors have been carried for many generations and studied thoroughly in various departments of this Institute. They were used after attaining a diameter of about 1 cm. The animals were sacrificed by decapitation, the tumors were dissected quickly, and after removal of necrotic portions, if any, the tumors were sliced with the Stadie-Riggs slicer (20). Usually two, three, or more tumors were pooled in order to obtain sufficient tissue (approximately 0.7 gm. dry weight) for an experiment. The ratios of fresh to dry weight were sufficiently close to 5.0 to use this value for calculation of the dry weights, except for the rat hepatoma, for which a ratio of 6.0 was found.

Incubation of tissue.—The sliced tissues were suspended in approximately 30 ml. of Ringer-phosphate solution (9) contained in Warburg-type flasks of 125 ml. capacity, carrying a side-bulb, a center well, and a 24/40 joint for attachment to regular manometers. For carbon dioxide absorption a filter-paper roll, soaked with 2 M CO_2-free sodium hydroxide, was placed in a small glass tube which fitted loosely into the center well. After attachment to the manometers the flasks were "gassed" with oxygen, equilibrated 5 minutes at 38°, and then shaken approximately 3 hours. Acid was then tipped in from the side-bulb to liberate bound CO_2; and, after shaking 5 minutes to allow complete absorption on the KOH-paper, the flask was removed from the manometer, and the tube plus filter paper quickly transferred to a stoppered flask containing freshly boiled water. The carbonate was precipitated by the addition of 1 M barium chloride solution and the precipitated barium carbonate filtered, dried, and transferred to planchets for counting. Data for normal tissues were obtained in exactly the same manner, except that skeletal muscle of rats and mice was used as Waring Blendor minces.

Isolation of radioactive quinidine citrate.—For isolation of citrate, experiments were conducted exactly as already described, except for the addition of 0.3 mM of citric acid to the medium as a trapping agent. At the close of the incubation, and after removal of bound CO_2, the solution was acidified strongly with sulfuric acid and extracted continuously with ethyl ether. After several hours extraction, the extract, which contained residual fatty acids, small amounts of ether-soluble acids such as succinic or fumaric, and all the aconitic acid added in those experiments in which it was used as an inhibitor, was discarded. This preliminary extraction greatly facilitated the subsequent isolation of pure quinidine citrate without undue loss of citrate. Extraction was then continued for 3 days, following which interval 0.3 mM of normal citrate was added to the aqueous solution and extraction continued 3 days longer. The combined ether extracts were evaporated, taken up in water, and precipitated with silver nitrate. The silver salt was repeatedly precipitated and redissolved to a constant specific activity. After removal of silver the citrate was precipitated as the quinidine salt (22) and the salt recrystallized to a constant specific activity.

Trans-aconitic acid, when used as an inhibitor, was dissolved just prior to its use, since it was found that on long standing of its solutions there is an appreciable isomerization to the *cis*-form. When such old solutions are used for inhibition purposes a large blank formation of citric acid is observed, owing to the action of aconitase on the *cis* acid. In those experiments in which citric acid was isolated after inhibition by *trans*-aconitate, 0.37 mM of carrier citric acid was added prior to extraction with ether. Otherwise the procedure was the same as just described.

RESULTS

Glucose oxidation.—A comparison of normal with tumor slices in their ability to oxidize glucose is given in Table 1. Little significance can be attached to the individual values given in this and subsequent tables, since they generally are obtained from single experiments. It is to be expected that individual tumors would display the same variations shown by normal tissue slices. What we wish to convey in these tables is the magnitude of oxidative capacity of a representative group of tumors for comparison with a representative group of normal tissues. The oxygen consumption rates of the neoplastic tissues were somewhat lower than, but similar in magnitude to those of most of the normal tissues. The respiratory quotients for the tumors were in the vicinity of 0.8, which is also within the usual range displayed by normal tissues. The column headed "R.S.A." gives the relative specific activity of the respiratory CO_2, a value which represents the percentage of glucose carbon in the total CO_2. The results indicate that relatively large portions of the total respiratory activity of kidney, brain, and heart were derived from glucose oxidation, whereas liver and skeletal muscle derived considerably less respiratory CO_2 from the added glucose. The capacity for glucose oxidation is displayed more directly in the last column in which values are given for the "oxidative capacity," a term which designates the micro-atoms of substrate carbon

converted to CO_2/gm dry tissue/hour. This value is the product of the R.S.A. (column 4) and the quantity of CO_2 (column 3). The normal tissues fall into two groups: kidney, heart, and brain, which oxidize glucose rapidly; and liver and skeletal muscle, which oxidize glucose more slowly. The rates of glucose oxidation in the four

TABLE 1

C^{14}-GLUCOSE OXIDATION BY NORMAL AND NEOPLASTIC TISSUE SLICES

All values are in μM/gm tissue dry weight/hour, unless otherwise specified

Tissue	O_2 μM	Respiratory CO_2 μM	R.S.A.* (per cent)	O.C.† micro-atoms carbon
Normal, rat:				
Kidney	660	560	19.9	111
Brain (homogenate)	291	277	34.2	93
Heart	354	325	26.8	87
Liver	362	278	8.5	23
Skeletal muscle (homogenate)	75	70	10.0	7.0
Neoplastic:				
Hepatoma (mouse)	308	242	16.5	43.8
Hepatoma (rat)	286	227	17.0	38.3
Rhabdomyosarcoma (mouse)	174	147	23.7	35
Mammary adenocarcinoma (mouse)	174	150	17.0	25.5

* The relative specific activity is the ratio of activity of CO_2 to substrate (× 100) and represents the relative amount of substrate carbon in the carbon of the CO_2.

† O.C. represents the micro-atoms of substrate carbon oxidized to CO_2 per gram dry tissue per hour at 38° at a substrate concentration of 0.005 M.

tumors fall between those of the two groups of normal tissues and exhibit a rather narrow range of variation. This observation substantiates previous conclusions of Greenstein (7) and others that, enzymically, tumors resemble one another more closely than do normal tissues.

Similar data for palmitate oxidation, given in Table 2, disclose that with the exception of kidney, which invariably has a much higher oxygen uptake, all the tissues, both normal and neoplastic, had about the same rates of oxygen consumption and CO_2 production, the same range of relative specific activities of respiratory CO_2, and approximately the same oxidative capacity.[1] These results leave no doubt concerning the capacity of neoplastic tissue to utilize fatty acids for energy production (1). A more detailed investigation of the oxidation of fatty acids in tumors is now under way and will be reported separately.

[1] The calculation of the oxidative capacity for the palmitic acid, as well as for the lactic acid used in this study, is predicated on the assumption that all the carbons are oxidized at the same rate as the labeled carboxyl carbon. Direct evidence for the validity of this assumption for palmitate is available from the work of Chaikoff *et al.* (16), who have shown that when palmitic acids labeled in various positions in the chain are oxidized by rat liver slices, the labeled carbon is converted to CO_2 at approximately the same rate.

The data of Table 3 on the oxidation of carboxyl-labeled lactic acid are of particular interest, because the high aerobic glycolysis of tumors might be presumed to reflect a disturbance in its oxidative removal. There was no substantiation for the concept that tumors as a class cannot oxidize lactic acid as readily as normal tissues. As with the other substrates, the range of variation of oxidative capacity among the different tumors was much narrower than that of the normal tissues. It is noteworthy that rat and mouse hepatoma oxidize lactate more readily than do the corresponding livers; and rhabdomyosarcoma oxidizes lactate more readily than skeletal muscle.

Participation of the citric acid cycle in tumors.—The capacity of tumor tissues for the complete oxidation of glucose and fatty acids having been

TABLE 2

PALMITATE OXIDATION BY NORMAL AND NEOPLASTIC TISSUES

Substrate concentration, 0.001 M

Tissue	O_2 μM	Respiratory CO_2 μM	R.S.A.	O.C.
Normal rat:				
Kidney	752	530	7.5	39.8
Liver (fasted)	367	265	7.0	18.6
Liver (fed)	307	244	4.1	10.0
Liver of tumor-bearing rat	314	224	3.9	8.7
Neoplastic:				
Hepatoma (mouse)	328	292	4.5	20.3
Hepatoma (rat)	260	184	7.4	13.6
Mammary adenocarcinoma (mouse)	308	250	4.4	11.0
Rhabdomyosarcoma (mouse)	257	246	6.7	16.5

TABLE 3

LACTIC ACID OXIDATION BY NORMAL AND TUMOR TISSUES

Substrate concentration, 0.005 M

Normal tissue	O.C.	Neoplastic tissue	O.C.
Rat liver	44	Rat hepatoma	61
" kidney	152	Mouse hepatoma	87
" heart	148	" Andervont hepatoma	48
" muscle	3	" Sarcoma 37	104
" brain	112	" rhabdomyosarcoma	72
" spleen	130	" mammary adenocarcinoma	65
Mouse liver	32	" Ehrlich ascites	116
" kidney	440		
" heart	108		
" muscle	5		

established by these isotopic studies, experiments were undertaken to determine whether, as in normal tissues, the citric acid cycle represents the pathway by which these oxidations occur. Saffran and Prado (18) found that *trans*-aconitic acid is an inhibitor of aconitase and observed that its addition to slices of liver and kidney cortex resulted in a marked decrease in oxygen consumption and

an increase in citrate accumulation. It was evident that both of these effects provide a reliable criterion for the occurrence of the citric acid cycle. The application of this procedure to slices of neoplastic tissue gave results which were, on the whole, quite similar to those observed by Saffran and Prado. With all three tumors studied in this manner, inhibitions of oxygen uptake and increases in citrate formation were observed (Table 4). Neither the increases in citrate nor inhibitions of oxygen consumption were as marked in tumors as in normal rat liver, but in every instance citrate accumulation and respiratory inhibition were observed in the presence of *trans*-aconitate. These results may be considered indicative of the occurrence of the citric acid cycle in the tumors; at the same time they suggest that the cycle may operate to a lesser extent than in normal tissues. However, quantitative deductions from such experiments are probably not justified.

To ascertain whether the citrate which accumulated in tumor slices in the presence of *trans*-aconitate did in fact arise directly by oxidation of added metabolites, two large-scale experiments were conducted with *trans*-aconitate with slices of mammary tumor: one in which labeled substrate carbon was provided in the form of C^{14} COOH-labeled palmitate, the other with COOH-labeled acetate. The citrate which accumulated in these experiments was isolated after addition of carrier citrate by continuous extraction with ether and conversion to the quinidine salt. Pure quinidine citrate was thus obtained, the radioactivity of which remained constant on repeated crystallization, indicating the formation of citrate from fatty acid carbon (Table 5). The observed activities of these samples were, of course, relatively very low, since the metabolic citrate represented only a small fraction of the isolated salt. For example, in the experiment with palmitate the relative specific activity of the citrate was 0.04 per cent. Carrier citrate (370 μM) was added, and, from the experiments in Table 4, we can estimate that about 2 μM of citrate would be expected to accumulate. The relative specific activity of this metabolic citrate therefore must have been (370/2) $\times$ 0.04 = $\sim$ 8 per cent. This value seems reasonable, since it is approximately the same as that of the respiratory CO_2 which presumably arose by oxidation of the metabolic citrate. Results were about the same with acetate as the substrate.

"Trapping" of metabolic citrate.—Subsequently, it was found that participation of citrate as an intermediary metabolite could be demonstrated by a "trapping" procedure. It was found that when nonradioactive citrate was incubated in 0.01 M concentration with tumor slices carrying out the oxidation of labeled substrates, the citric acid became labeled, owing to mixing of the metabolically formed isotopic citrate with the extracellular nonisotopic citrate. In Table 6 there are listed the results of such experiments in which C^{14}-labeled glucose was employed as the substrate. In every instance pure, radioactive quinidine citrate was obtained. The activities were low compared to those of the starting materials, but this was not unexpected, since it was anticipated that only minute portions of the metabolic citrate would be "trapped" in the large bulk of extracellu-

TABLE 4

INHIBITION OF RESPIRATION AND ACCUMULATION OF CITRATE CAUSED BY *Trans*-ACONITATE

Experiments run 2 hours in O_2 at 38° in a calcium-free Krebs-Ringer solution buffered at pH 7.4. Values are given in micromoles per gram dry tissue per hour

Substrate	*Trans*-aconitate	Tissue: Rat liver Oxygen	Rat liver Citrate	Rhabdomyosarcoma Oxygen	Rhabdomyosarcoma Citrate	Mammary carcinoma Oxygen	Mammary carcinoma Citrate	Hepatoma Oxygen	Hepatoma Citrate
None	−	290	0.85	180	1.2	308	1.4	350	2.1
"	+	210	6.2	116	2.4	240	2.4	270	3.7
" (in N_2)*	+				0.8		0.9		1.5
Malate	−	390	3.0	220	2.3	405	1.6	270	2.1
"	+	240	20.5	190	4.3	325	2.8	260	6.3

* This serves as a blank value, giving the maximum amount of citrate which is present in the tissue, as such, or is formed by the action of aconitase on any *cis*-aconitate present in the *trans* acid.

TABLE 5

ACCUMULATION OF RADIOACTIVE CITRATE IN PRESENCE OF *Trans*-ACONITATE

Each flask contained freshly prepared *trans*-aconitate in 0.02 M concentration

Substrate	Respiratory CO_2 (μM)	Respiratory CO_2 R.S.A.	Citrate Relative specific activity, per cent: Observed	Citrate Relative specific activity, per cent: Corrected for carrier
Acetate, 0.01 M	460	5.4	0.03	~6
Palmitate, 0.01 M	370	9.2	0.04	~8

lar citrate. Oddly, the normal tissues, heart and liver, displayed a much lower incorporation of glucose carbon into citrate than did the tumor slices. This is probably due to preferential oxidation of citrate by these tissues, since the isotope data showed that glucose oxidation was very low in these experiments. Similar experiments with hepatoma and rhabdomyosarcoma, also given in Table 6, demonstrate, by the same method, the intermediary formation of citrate from palmitate.

TABLE 6

RADIOACTIVE CITRATE FROM TUMOR OXIDATIONS

Tissue	Substrate	Specific activity (counts/min)	Quinidine citrate specific activity (counts/min)
Normal, mouse:			
Heart	Glucose	5.5×10^5	150
Liver	"	"	126
Kidney	"	"	2,480
Neoplastic, mouse:			
Hepatoma	"	1.37×10^6	1,225
Mammary tumor	"	"	1,750
Rhabdomyosarcoma	"	5.5×10^5	1,000
Ehrlich ascites	"	"	910
Mammary tumor	Acetate	1.4×10^6	293
" "	Palmitate	66,200	74
Hepatoma	"	"	138
Rhabdomyosarcoma	"	"	83

DISCUSSION

It is generally agreed that the catabolism of glucose in normal cells involves an anaerobic breakdown to 3-carbon acids, followed by an oxidative phase in which pyruvic acid is completely oxidized by way of the citric acid cycle. Recent studies of Meyerhof and Wilson (10) and Novikoff, Potter, and LePage (11) have established that the glycolytic phase proceeds by the same pathway in tumors as in normal tissues. The present study supplements these investigations by demonstrating the capacity of tumor cells to oxidize glucose to completion, and by demonstrating that the oxidative phase of glucose catabolism proceeds also in tumors *via* the citric acid cycle. This study thus provides no basis for the concept that oxidation of carbohydrate or fatty acid is qualitatively different in tumors from that in normal cells. Since the oxidative capacity for glucose and palmitic acid (and also lactic acid) displayed by tumor slices was in the range of that of a series of normal tissue slices, one may assume that quantitatively also tumor cells are similar to normal ones in their capacity to carry out oxidations *via* the citric acid cycle.

Since these findings appear at first sight to be at variance with some of the conclusions of certain recent studies of tumor metabolism, a brief discussion of this matter is in order. Several lines of evidence have been cited by Potter and coworkers to indicate that there is a relative or absolute deficiency of oxidative metabolism in tumors (12, 14). The most direct evidence bearing on this point is that certain enzymes such as malic dehydrogenase, DPN-cytochrome c reductase, succinic dehydrogenase, and cytochrome oxidase (13, 17, 19) occur in relatively lower amounts in tumors than in such normal tissues as liver, kidney, and brain. Our data are not in direct conflict with these findings, since it is conceivable that certain enzymes may be decreased considerably in tumors without manifesting changes in the overall respiration rate of the tissue slice.

There is an element of uncertainty in the assay of oxidative enzymes which has to be considered, however. Two instances may be cited in which assays of dehydrogenases by manometry apparently give falsely low values. Potter and co-workers (13, 17) reported malic dehydrogenase and DPN-cytochrome c reductase to be quite low in tumors. In unpublished work to be reported later Wenner, Spirtes, and Weinhouse found malic dehydrogenase to be as high in tumors as in normal tissues when assayed by a spectrophotometric method based on oxidation of reduced DPN and which is hence independent of other electron transport factors. Recently, Hogeboom and Schneider (8), also using a spectrophotometric procedure, found *increased* DPN-cytochrome c reductase in hepatoma as compared to liver. In the manometric assay of these enzymes, some factor involved in electron transport rather than the dehydrogenase under assay may be limiting the rate of oxygen uptake.

Another instance of the apparent failure of tumor tissue to carry out reactions of the citric acid cycle is the almost complete absence of oxalacetate oxidation in fortified homogenates of Flexner-Jobling carcinoma, reported by Potter, LePage, and Klug (16). It was established that this was not due to an inability of the tumor tissue to maintain its adenosine triphosphate (ATP) level, and it was tentatively assumed that the tumor lacked the enzyme necessary for condensation of oxalacetate to citrate (15). However, this "condensing" enzyme has been found in amounts comparable to those of normal tissues in a variety of transplanted mouse tumors (24); and inasmuch as other enzymes of the Krebs cycle were found present in amounts which are in the range of normal tissues (24), the failure of oxalacetate to be oxidized by tumor homogenates might have been caused by loss of some labile factor during the homogenization process. It was found that this

explanation is at least partially correct, since in unpublished work Wenner, Spirtes, and Weinhouse found that the addition of diphosphopyridine nucleotide (DPN) in relatively high concentration (.001 M) restored the capacity of various mouse tumor homogenates for oxalacetate and pyruvate oxidation. Whether other factors are also involved and whether this effect of DPN is due to an initially low level of the nucleotide or to its more rapid breakdown in broken tumor cells are subjects for further investigations.

SUMMARY

The oxidation of isotopically labeled glucose, lactic acid, and palmitic acid was studied in slices of transplanted tumors by measurement of incorporation of radioactivity in the respiratory carbon dioxide. It was found that the oxidation of these substances proceeded in tumors at rates within the range of a representative group of normal tissues. Participation of the citric acid cycle in the oxidation of glucose and palmitic acid was shown by inhibition experiments with *trans*-aconitate and by the formation of radioactive citric acid as an intermediate. It was concluded that the oxidation of carbohydrate and fatty acid by intact tumor cells is qualitatively and probably also quantitatively similar to that of normal tissues.

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Subcutaneous Implantation of Whole Mouse Pituitary and the Estrous Behavior of Ovariectomized C3H Mice*

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Since the work of Lathrop and Loeb (6), it has been apparent that certain ovarian hormones are necessary to the production of mammary carcinoma in mice. Subsequent studies have shown that estrogenic substances are of primary importance. The unexpected observation of Woolley, Fekete, and Little (7) that following ovariectomy in certain strains of mice there occurred a high incidence of mammary carcinoma, associated with adrenal cortical adenomas, raised the suggestion that under such circumstances the adrenal cortex may produce estrogen. This inference is supported by the fact that such mice show vaginal smears indicating a state of continuous subestrus.

Casas, King, and Visscher (1, 2) found that caloric underfeeding of ovariectomized C3H mice did not prevent the development of adrenal cortical hyperplasia and metaplasia but did inhibit the development of the estrous state (5) and did prevent mammary cancer development. They inferred that caloric underfeeding prevented, by direct or indirect means, the production of estrogen by the adrenal gland. These workers[1] placed ovariectomized mice on a diet which restricted caloric intake by one-third, while maintaining the usual intake of protein, minerals, and vitamins. These mice remained anestrous; at the age of 6 months they were placed on ad libitum feeding, and all the mice developed a dense mixed (subestrous) smear in from 14 to 36 days, with a mean of 20.4 days. When these mice once came into subestrus, they remained in subestrus as long as they were on ad libitum feeding.

Since caloric restriction is thought to cause inhibition of the pituitary, particularly as to the output of gonadotropic hormone, the injection of pregnant mare serum gonadotropin into such ovariectomized calorie-restricted mice was tested by King (4) *et al.* The effect of injection of corticotropin under these same conditions was tried with similar results by these same authors (3). Such treatment did not induce estrus. This result is in contrast to the stimulation of estrogen production induced by gonadotropin in calorie-restricted intact mice. The latter are also in an anestrous state but go into the estrous state upon administration of gonadotropin. A similar result occurs following implantation of whole mouse pituitaries (one daily) for 4 days in intact calorie-restricted mice.[2]

In view of these findings it seemed important to ascertain whether pituitary implantation in anestrous C3H mice following ovariectomy and a period of caloric underfeeding would hasten the appearance of subestrus.

MATERIALS AND METHODS

Thirty-two female C3H mice born within a 7-day period were ovariectomized at 21–25 days of age; when 5 weeks old they were placed on a diet which restricted their caloric intake to two-thirds that of control C3H mice on an ad libitum diet, while maintaining their intake of protein, minerals, and vitamins at a normal level. The food was measured by pressing out a pellet by means of a standardized brass mold machined to contain the specified amount of this particular diet. Throughout the experiment the mice were housed individually in wire and sheet-metal cages 20×15×15 cm. and were given water ad libitum. Vaginal smears were studied from the age of 204 days (plus or minus 3 days) for 5 days, and at several irregular times before that to detect signs of estrogenic stimulation. At the age of 212 days (plus or minus 3 days) the first twenty of the mice then living were taken off their restricted diet and were placed on an ad libitum control diet. (See Table 1 for the composition of these two diets.)

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[1] Unpublished data.

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[2] Unpublished data.

The twenty mice were divided into two groups of ten mice each, with the mouse weights in the two groups matched as closely as possible. Group I received daily subcutaneous implantations of one whole mouse pituitary obtained immediately after sacrifice under ether from a sexually mature, but not senescent, C3H strain female mouse; after 31 days the supply of C3H donor mice was exhausted, and A strain female mice were used for donors for the balance of the implantations, which were carried out for a total of 46 days. Group II received daily subcutaneous implantations of a comparable amount of gastrocnemius muscle obtained from a C3H or A strain female mouse. All implantations were inoculated subcutaneously into the backs of the recipient mice immediately after the material was obtained from the donor mice.

TABLE 1

DIETS FED TO AD LIBITUM AND CALORIE-RESTRICTED ANIMALS

Component	Ad libitum diet (gm/100 gm)	Restricted diet (gm/100 gm)
Glucose	34.02	22.91
Lard	17.53	12.81
Casein	28.87	38.95
Dry yeast	8.25	11.10
Dry alfalfa leaf	4.12	5.62
Salt mixture (Jones-Foster)*	4.12	5.54
Cod liver oil	2.06	2.08
Wheat germ oil	1.03	1.04

* Parts by weight to make up Jones-Foster salt mixture are as follows: NaCl, 585; KH_2PO_4, 1,633; $MgSO_4$, 241; $CaCO_3$, 1,602; $FeSO_4$, 62; KI, 3.32; $MnSO_4 \cdot H_2O$, 16.9; $ZnCl_2$, 1.09; $CuSO_4$, 1.28; $CoCl_2 \cdot 6H_2O$, 0.095.

Daily vaginal smears were made on all the mice for the first 59 days after the full feeding, and the implantation of pituitary glands was instituted and at intervals of from 1 to 6 days thereafter. Physiological saline and a small pipet were used for washing out the vagina to obtain the vaginal smears. It was felt that by this method daily vaginal smears could be obtained while avoiding cornification of the vagina that is often found when other methods of obtaining vaginal smears are used. If at least one-third of the cells in a smear were epithelial or cornified epithelial cells, and if the smear was dense, it was considered to be a subestrous smear. The weight and food intake of the mice were determined each week. Pituitary implants were discontinued after being given daily for 46 consecutive days.

RESULTS

At the start of full feeding and implants, and for 3 weeks thereafter, the average weight of the mice in the two groups was the same; thereafter, the weight of mice in the control Group II averaged approximately 1 gm. less than that of the mice in the experimental Group I. Also, in Group II, the intake of food after the second week was approximately 2.5 gm. less per mouse per week than in Group I (Chart 1).

All the mice were in anestrus during the period of caloric restriction. During the 148-day period after implantation and full feeding were instituted, four mice in Group I and nine mice in Group II came into subestrus. During this 148-day period mice in the experimental Group I were in subestrus for 49 days, and mice in the control Group II were in subestrus for 116 days (Chart 2). There was thus more than a twofold difference between the two groups. After the first 59 days, vaginal smears were taken only at irregular intervals. It was thought that any effects from the implanted pituitaries would show up during the time of implantation or during the 2 weeks after implants were discontinued.

One mouse in Group I had a change in vaginal smear from the anestrous to subestrous type on the thirteenth day after implants and full feeding were instituted; the first subestrous smear in Group II was noted on the fifteenth day. Mice in Group I were in subestrus for 14 of the 46 days during which implantations were made; mice of Group II were in subestrus for 28 days. Mice of Group I were in subestrus for 17 of the 59 days during which smears were taken daily as compared to the mice of Group II which were in subestrus for 43 days (Chart 2).

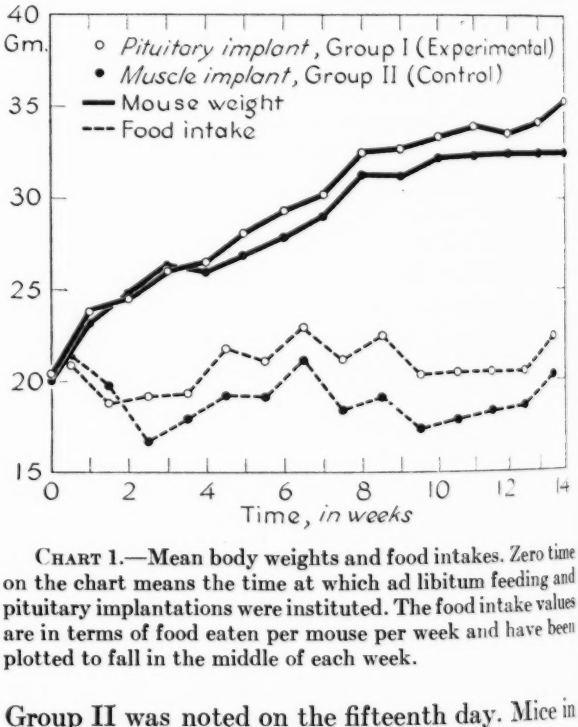

CHART 1.—Mean body weights and food intakes. Zero time on the chart means the time at which ad libitum feeding and pituitary implantations were instituted. The food intake values are in terms of food eaten per mouse per week and have been plotted to fall in the middle of each week.

It may be noted that most of the subestrous smears in the experimental Group I were contributed by one of the ten mice in the group during the first 59 days after full feeding and implants were begun. After the first 59 days, several of the mice in the group began to have subestrous smears quite regularly. A similar situation existed in the control Group II.

One hundred forty-eight days after reversal to full feeding, all the mice in both experimental and control groups then living were sacrificed, and a post mortem examination was carried out. In none of these mice, nor in any that had died while the experiment was being carried out, was an abscess found. The sites of subcutaneous implantation were searched with special care.

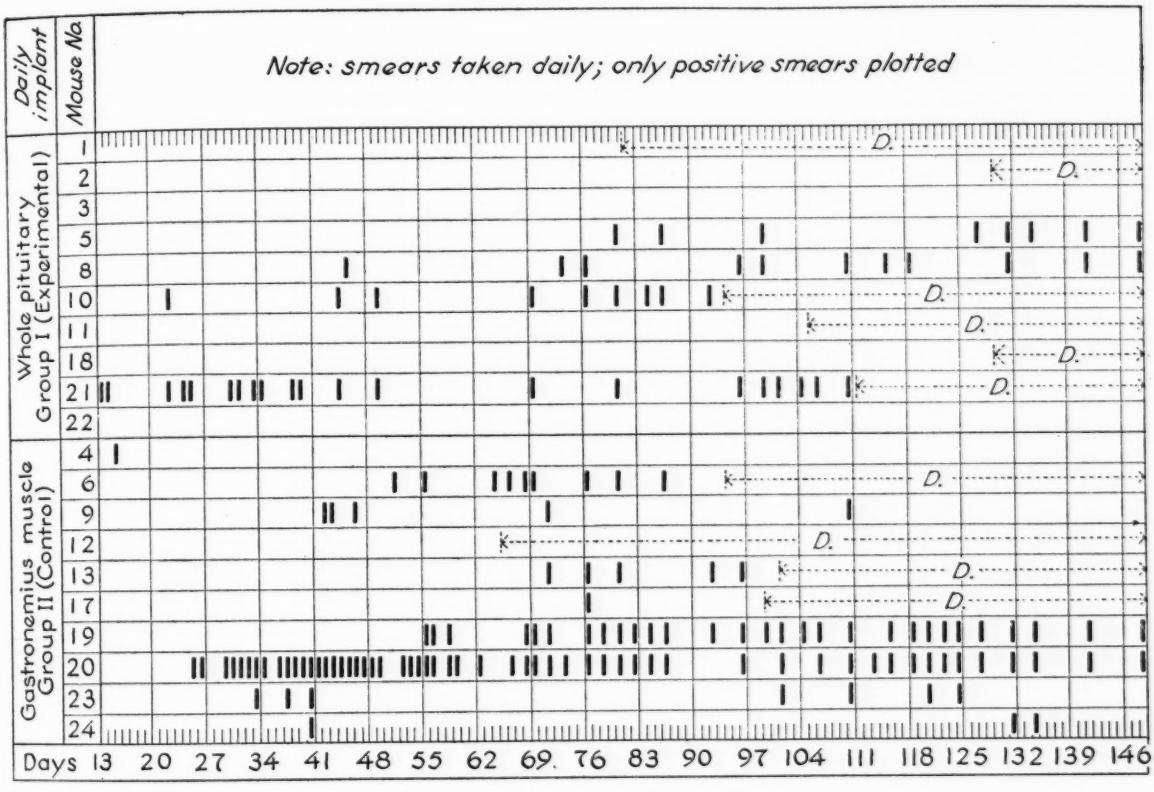

CHART 2.—Estrous behavior of ovariectomized mice with whole pituitary implantations. The chart indicates the subestrous smears found for each mouse in the control and experimental groups during the period of the 13th day through the 148th day, inclusive, after pituitary implantations and ad libitum feeding were instituted. No mice were in subestrus the first 12 days. The pituitary implantations were given daily from the 1st through the 46th day, inclusive. After the 59th day, smears were made only at irregular intervals, and thereafter the data indicate only the difference in frequency of subestrous smears between the groups on particular days. The letter *D* indicates the time of death of a mouse.

DISCUSSION

This experiment was set up to test the hypothesis that castrated female mice on caloric restriction fail to show estrus because of a functional hypopituitary state induced by the underfeeding. Since such mice ordinarily enter a subestrous state within a month after being placed on ad libitum feeding, it seemed that if pituitary hypofunction were the limiting factor, the time for appearance of estrus might be reduced by pituitary gland implantation. This view was supported by the fact that the anestrous calorie-restricted intact mouse is brought into full estrus by four daily implantations of a whole mouse pituitary. It is recognized, of course, that ovarian stimulation and adrenal cortex activation, even in the production of estrogen, may be mediated by quite different mechanisms, but it appeared that the hypothesis deserved thorough testing.

The results show no confirmation of the hypothesis that failure of adrenal cortical tissue to produce estrogens in calorie-restricted ovariectomized mice is due to pituitary hypofunction. These findings do not indicate that caloric restriction does not cause hypophyseal inhibition, but they do suggest that another probably more direct effect of underfeeding upon the adrenal gland is more crucial, especially when taken in conjunction with previous findings that gonadotropin (4) or corticotropin (3) is without effect in these animals.

The fact that the controls with muscle implants exceeded the mice with pituitary implants in frequency of the subestrous smear is difficult to interpret. The difference is statistically significant at the 1 per cent level. Pituitaries from mice of the same (highly inbred) strain were used for the major part of the experiment with the definite purpose of avoiding foreign protein and anti-hormone reactions. Transplants of tissue from one animal to another within such strains give persistent growth. It is therefore to be doubted that anti-hormones account for the results. The body weight gain and food intake were greater in the pituitary-implanted than in the muscle-implanted group. In general, one would have expected the former group rather than the latter to have shown the earlier appearance of estrus on a weight gain basis alone. It is possible that certain active agents in whole pituitary are inhibitory with respect to the over-all process involved in estrogen production by adrenal cortical adenomas in previously underfed mice.

SUMMARY AND CONCLUSIONS

The implantation of whole mouse pituitaries into ovariectomized C3H mice on full feeding following a period of caloric restriction did not result in an acceleration of onset of the subestrous state characteristic of such mice. In fact, control mice implanted with gastrocnemius muscle showed a somewhat greater tendency to reach the subestrous state at an earlier time. These results lend no support to the hypothesis that pituitary hypofunction in caloric underfeeding is the critical limiting factor in preventing estrogen production in ovariectomized mice with resulting adrenal cortical tumors.

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Studies on the Ehrlich Ascites Tumor

I. The Enzymic and Metabolic Activities of the Ascitic Cells and the Ascitic Plasma

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The Ehrlich mouse ascites tumor (Loewenthal and Jahn [19]) offers many advantages over solid transplantable tumors as a tool for the study of the growth and biochemistry of malignant tissue. It provides a readily obtainable and almost pure culture of discrete tumor cells, virtually uncontaminated by blood, connective tissue, and necrotic material. Although some information concerning the concentration of nucleic acids (14, 15) in these tumor cells is extant, practically nothing is known of their metabolic and enzymic activities. Prior to investigating the biochemical and nutritional factors which determine the growth of this tumor both *in vivo* and *in vitro*, we have examined the metabolic characteristics of the intact tumor cells as well as the enzymic properties of tumor homogenates and cytoplasmic particles isolated from the latter by differential centrifugation. Some studies on the chemical composition and enzymic activities of the fluid (which may be conveniently termed the "ascitic plasma") bathing these cells in the intact mouse are also included in this communicati·n.

METHODS

Determination of the respiration, glycolysis, and respiratory quotient of intact tumor cells.—The ascitic fluid was removed from the mice immediately following death by cervical fracture and was centrifuged at once for 3–5 minutes at 2,000 $\times g$. The resulting supernatant fluid (the ascitic plasma) was decanted, and the tumor cells were scooped out, leaving a small layer of erythrocytes at the bottom of the tube. The tumor cells were transferred to another tube and washed twice with 3 volumes of the appropriate suspension medium. After the second washing, the cells were resuspended in the medium and cell counts made in an hemocytometer. The cell suspension was then diluted so as to contain a known convenient number of cells. For respiration and respiratory quotient experiments a suspension was used containing approximately 5×10^7 cells/ml, while in determinations of aerobic and anaerobic glycolysis suspensions containing approximately 2.5×10^7 cells/ml were employed. The cells suspensions were stored at 2° for not more than 2 hours prior to use.

Respiration experiments were conducted with Krebs-Ringer phosphate buffer of pH 7.4, while in glycolysis experiments the medium was either the latter or Krebs-Ringer bicarbonate, calcium chloride being present in both media (30).

The respiration of the cells was determined manometrically with 0.20 ml. of 10 per cent KOH and filter paper roll in the center well of each Warburg flask. Each flask contained 1.70 ml. Krebs-Ringer phosphate buffer of pH 7.4, 0.30 ml. water and/or additions, and 1.00 ml. cell suspension. The gas phase was either air or 100 per cent oxygen. After 10 minutes of temperature equilibration, oxygen uptakes were measured at 10–30-minute intervals during the course of the experiment. In experiments where the influence of NaCN on the respiration of the cells was studied, the appropriate KOH-NaCN mixtures (17) were added to the center well.

Anaerobic glycolysis was determined manometrically in Krebs-Ringer bicarbonate solution with 95 per cent N_2-5 per cent CO_2 as the gas phase. Each flask contained 1.70 ml. Krebs-Ringer bicarbonate, 0.30 ml. water and/or additions, and 1.00 ml. cell suspension. In some cases the manometric determinations were checked by chemical determinations of the lactic acid present in the flasks at the beginning and end of the experiment. Aerobic glycolysis was measured by lactic

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acid determinations using the same set-up employed for the determination of respiration. In all glycolysis experiments the substrate was tipped into the main compartment at the end of the temperature equilibration period.

Respiratory quotients were determined by the direct method (30), CO_2 retention at the beginning and end of the run being measured by tipping 0.20 ml. 5 N H_2SO_4 from the side-arm into the main compartments of the appropriate flasks.

All measurements of respiration, glycolysis, and respiratory quotients were conducted at 37°.

Determination of hexokinase activity.—Acetone powders prepared from washed tumor cells were triturated with 30 volumes of ice-cold water and allowed to stand for about 1 hour at 2°. The suspensions were then centrifuged at 2° for 15 minutes at 2,000×*g;* 0.50 ml. of the resulting supernatant fluid was added to each flask as a source of the enzyme. Hexokinase activity was determined manometrically according to the principles of Colowick and Kalckar (3). In addition to the enzyme, the main compartment of the flasks contained 1.50 ml. 0.04 M $NaHCO_3$, 0.3 ml. 0.5 M NaF, 0.20 ml. 0.1 M $MgCl_2$, and 0.20 ml. water and/or sugar. The side-arm contained 0.10 ml. 0.04 M $NaHCO_3$ and 0.20 ml. K-ATP (containing 30 μM acid-labile P). The flasks were gassed with 95 per cent N_2-5 per cent CO_2; after 10 minutes equilibration at 37°, the contents of the side-arm were tipped into the main compartment, and the CO_2 evolution measured at 10-minute intervals. Blanks containing all the reagents except sugar were run, to correct for the CO_2 evolution due to the acidity or alkalinity of the ATP on tipping and also the ATP-ase activity of the preparation (which was negligible). The CO_2 outputs recorded are corrected for such blank determinations.

Determination of DPN-ase.—The reaction was carried out in open test tubes, each containing 0.50 ml. 0.05 M glycyl-glycine buffer of pH 7.5, 0.07 ml. 2 M KCl, 0.68 ml. water, and 0.50 ml. enzyme suspension. The tubes were incubated for 10 min. at 22°, being shaken to and fro 150 times per minute, prior to the addition of 0.15 ml. of a solution containing c. 2.5 μM DPN. Appropriate tubes were immediately deproteinized by the addition of successive equal volumes of $Ba(OH)_2$ and $ZnSO_4$ of equivalent normality to give values for the initial DPN concentration. The reaction was allowed to proceed for 60 minutes, after which time the tubes were similarly deproteinized. The DPN and the nicotinamide-ribose moiety of the latter and/or its breakdown products and also the free nicotinamide present in filtrates of the deproteinized reaction mixtures were then determined.

Determination of oxidative phosphorylation catalyzed by ascitic tumor particles.—"KCl particles" were prepared by homogenizing the cells, previously washed twice, in approximately 5 volumes of ice-cold 0.15 M KCl, to which sufficient solid $NaHCO_3$ had been added to adjust the pH to 7.8. The homogenate was centrifuged at 2,000×*g* for 30 min. at 0°. The resulting supernatant fluid was discarded and the precipitate washed twice with KCl. The final precipitate was suspended in KCl, 1.00 ml. of the suspension being added to each flask.

Mitochondria were isolated from homogenates prepared in 0.25 M sucrose according to the procedure of Hogeboom, Schneider, and Pallade (8). The mitochondrial pellet was washed twice with this medium and finally suspended either in 0.25 M sucrose or in 0.15 M KCl.

Measurements of the P/O ratio associated with succinate oxidation were conducted by use of methods similar to those described by Judah and Williams-Ashman (11). The final volume of the reaction mixture was 3.00 ml. and contained the following components: glycyl-glycine buffer of pH 7.5, 50 μM; KF, 40 μM; K-phosphate of pH 7.5, 10–20 μM; ATP, 6 μM; fructose, 111 μM; cytochrome c, 0.12 μM; $MgCl_2$, 20 μM; succinate, 100 μM; and 0.20 ml. yeast hexokinase. When the mitochondria were suspended in sucrose, an additional 150 μM KCl was added to each flask. The reaction was stopped by the addition of 0.20 ml. of 100 per cent trichloroacetic acid from the side-arm, and the deproteinized reaction mixture was diluted with a further 3.0 ml. 10 per cent trichloroacetic acid prior to filtration.

Determination of other enzymes.—Acid and alkaline phosphatase activities were determined at pH 5.0 and pH 9.3, respectively, with disodium monophenyl phosphate as a substrate, by a modification of the method of King and Armstrong (13). β-Glucuronidase activity was measured by the method of Talalay, Fishman, and Huggins (29). Succinoxidase activity was determined according to Schneider and Potter (27) and malic dehydrogenase by the method of Potter (25), with the exception that in both cases the homogenates were prepared in ice-cold 0.15 M KCl (1.00 ml. being added to each flask). Aldolase activity was determined by the method of Sibley and Lehninger (28), and the enzymic conversion of 3-phosphoglycerate to phosphorpyruvate was estimated according to Kun (18). Choline esterase activity was determined according to the principles described by Mendel, Mundell, and Rudney (22). The final concentrations (in a final volume of 2.5 ml.) of the reagents used for choline esterase determinations

were: $NaHCO_3$, 0.029 M; acetylcholine, 0.034 M; acetyl-β-methyl-choline, 0.039 M; the gas phase, 95 per cent N_2-5 per cent CO_2. Blank vessels containing all the reagents but without enzyme were set up in order to obtain values for the rates of nonenzymatic hydrolysis of the substrates under these conditions. All values recorded are corrected for such blank determinations.

Preparations.—Yeast hexokinase was prepared according to the method of Berger *et al.* (2), the preparation being carried as far as stage 3*a*. The resulting precipitate was dissolved in 10 per cent fructose and stored in solution at −25°. Crystalline yeast alcohol dehydrogenase was isolated by the method of Racker (26) and either stored in aqueous solution at −25°, or freeze-dried with 99 parts of serum albumin and stored as a dry powder at 0°. Diphosphopyridine nucleotide (DPN) was obtained from Sigma Chemical Co., St. Louis, Mo., and further purified by counter-current distribution (7). The products obtained were assayed enzymically with yeast alcohol dehydrogenase in pyrophosphate buffer of pH 9.0, the purity of various samples ranging from 71 per cent to 74 per cent. Adenosine triphosphate (ATP) was obtained as the dibarium salt from Armour and Co., Chicago, Ill. The inorganic P content of the preparations used was always less than 8 per cent of the total acid-labile P (determined after hydrolysis in 1 N HCl for 10 minutes at 100°). Hexose diphosphate (HDP) was obtained as the dibarium salt and purified chromatographically on Amberlite resins.[1]

Acetone powders were prepared from tumor cells washed twice with cold, isotonic NaCl. The cells were comminuted in a chilled Waring Blendor with 3 volumes of acetone (−25°). The insoluble material was filtered off on a Buchner funnel and washed 5 times with cold acetone, without allowing the filter cake to crack. The powder was dried over P_2O_5 and stored at 2° *in vacuo*.

Homogenates were prepared with the aid of an all-glass homogenizer.

Estimations.—Glucose was determined in Ba-Zn filtrates by the method of Nelson (24); inorganic ortho-P either by the method of Gomori (6) or that of Fiske and SubbaRow (4); lactic acid in copper-lime filtrates by the method of Barker and Summerson (1); and phenol by the method of Folin and Ciocalteu (5). Nitrogen analyses were kindly performed by Mr. W. Van Epps, using the Kjeldahl procedure. The protein content of the ascitic plasma was determined in two ways: (*a*) by multiplication of the difference between the total N and the nonprotein N by the factor of 6.25 and (*b*) spectrophotometrically from the absorption of 260 mμ and 280 mμ in 0.033 M phosphate buffer of pH 7.4, using the formula and parameters given by Kalckar (12). DPN was assayed spectrophotometrically at 340 mμ after being converted to $DPNH_2$ by an ethanol-yeast alcohol dehydrogenase system. The reaction was carried out in a final volume of 3.00 ml. containing 50 μM sodium pyrophosphate buffer of pH 9.0, 1 mM ethanol, and sufficient alcohol dehydrogenase to bring the reaction to equilibrium within 2 minutes. The DPN concentration was computed with the extinction coefficient for $DPNH_2$ given by Horecker and Kornberg (9). The nicotinamide-ribose moiety of DPN and/or its breakdown products were determined fluorimetrically (10), and nicotinamide was determined spectrophotometrically (21). Absorption spectra were determined with the aid of a Beckmann DU quartz spectrophotometer.

[1] We wish to thank Dr. J. A. Sicé for the purification of this material.

RESULTS

Biological Data

The Ehrlich ascites tumor was kindly supplied to us by Dr. Kanematsu Sugiura, Sloan-Kettering Institute for Cancer Research, New York, and is the same tumor as that used by Klein (14). The tumor was maintained by approximately weekly intraperitoneal inoculations of whole ascitic fluid in hybrid white mice of both sexes (Carworth Farms, CF1 strain) and in a few instances in AKM strain mice (Jackson Memorial Laboratories, Bar Harbor, Maine). The mice were 2–5 months of age and were maintained on a standard pellet diet (Derwood Mills Mouse and Rat pellets) with adequate drinking water.

The rate of accumulation of ascitic fluid, length of life of the mice, and regression of the tumor are, within limits, functions of the number of inoculated cells and the strain of mouse. For practical purposes, intraperitoneal inoculations of 0.1 ml. of 7-day-old ascites per mouse provide a convenient method of growing this tumor, and there is almost 100 per cent susceptibility to this tumor in the mouse strains mentioned. For this work, all the tumor fluids were between 7 and 12 days old. Characteristically, the fluid is milky white, has a tendency to clot, and occasionally is grossly hemorrhagic. Fluids containing more than about 15 per cent erythrocytes of the total cells were discarded. All determinations were made with pooled samples derived from several mice.

The cells are of an apparently uniform histological type and contain multiple characteristic refractile vacuoles. Histochemical examinations

demonstrated that they are very rich in ribonucleic acid content and contain small amounts of neutral fat.[2] A cell block preparation of this tumor is shown in Figure 1.

The Metabolic Activities of Washed Intact Ascitic Tumor Cells

Anaerobic glycolysis.—In the absence of added substrate, the CO_2 production and chemically determinable lactic acid formation in the presence of bicarbonate buffer in equilibrium with 95 per cent N_2-5 per cent CO_2 was negligible. High rates

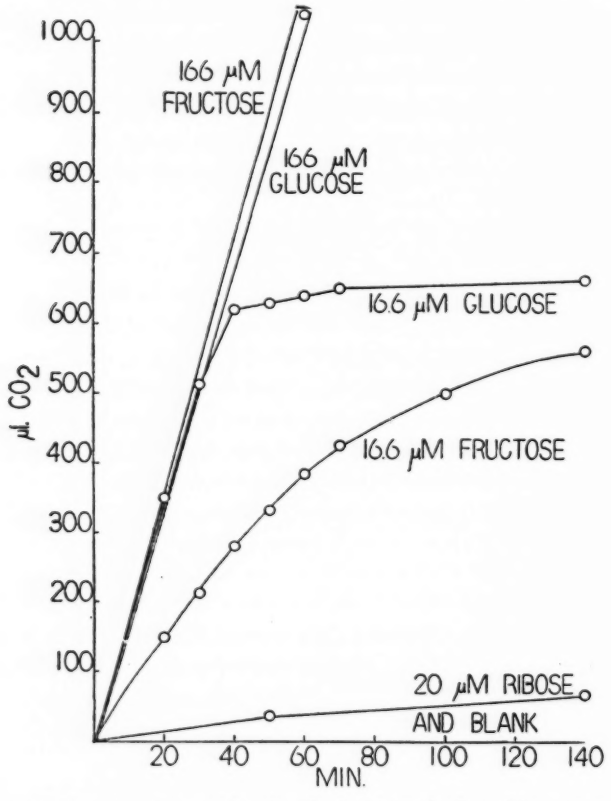

Chart 1.—Anaerobic glycolysis of washed ascitic tumor cells. Each vessel contained 4×10^7 cells.

of anaerobic glycolysis were observed, however, in the presence of either glucose or fructose. Provided they were present in excess, the rate of anaerobic glycolysis of these sugars was linear over an initial period of at least 3 hours. The absolute rates varied somewhat from one preparation to another, the mean $Q^N_{CO_2}$ (μl CO_2/mg dry weight cells/hour) and $Q^N_{CO_2}$ (N) (μl CO_2/mg N/hour) for 6 determinations of different pooled samples being 48.2 and 341, respectively. CO_2 production, approximately equal to lactic acid formation, was also observed when methylglyoxal (0.04 M) was tipped into the main compartment, but the rate was maintained for only a short while and decreased rapidly after 20 minutes, long before the theoretical amount of lactic acid was produced. Values for the CO_2 production obtained with various sugars were in excellent agreement with lactic acid determinations performed on copper-lime filtrates of the suspension medium.

When present in high concentration (0.055 M), fructose was glycolyzed 2–10 per cent faster than the same concentration of glucose. At lower concentrations of sugar, however, marked differences in the rate of glycolysis of glucose and fructose were observed. Chart 1 shows that 0.0055 M glucose was glycolyzed at practically the same rate as 0.055 M glucose until more than 80 per cent of the theoretical CO_2 was evolved, at which time the rate with the lower concentration of glucose rapidly fell to zero. 0.0055 M Fructose, however, was glycolyzed at only about 40 per cent of the rate of 0.055 M fructose over an initial 30-minute period. D-Ribose gave rise to a CO_2 output no greater than that in the absence of added sugar.

Janus green 6B (5×10^{-5} M) and methylene blue (5×10^{-4} M) had no significant effect on anaerobic glycolysis in the presence of glucose; 2,4-dinitrophenol (5×10^{-5} M) caused a small and variable activation (up to 40 per cent).

Aerobic glycolysis.—Determinations of the aerobic glycolysis in phosphate-buffered Krebs-Ringer showed that the rate of lactic acid formation in the presence of high concentrations of glucose was approximately 70 per cent of that observed in the presence of Krebs-Ringer bicarbonate in equilibrium with 95 per cent N_2-5 per cent CO_2 at the same pH. Thus, in a typical experiment, 2.5×10^7 cells catalyzed the formation of 19.7 μM lactic acid in 1 hour under anaerobic conditions; while aerobically 14.3 μM lactate were produced, the lactic acid formation in either air or nitrogen and in the absence of added sugar being nil.

Respiration.—In the presence of Krebs-Ringer phosphate of pH 7.4, a marked rate of respiration was observed in the absence of any added substrate. The mean absolute values for this endogenous respiration in nine experiments were: Q_{O_2} = 8.1; Q_{O_2} (N) = 58.5. The rate of the endogenous respiration was linear with time for about the first 60 minutes, after which interval it gradually declined. After 11 hours, the respiration rate was still approximately 25 per cent of the initial rate.

Both glucose and fructose invariably depressed the rate of oxygen uptake. The inhibition produced by a final 1 per cent concentration of these sugars was observed from the beginning of the experiment and varied with different preparations from 40 per cent to 60 per cent; but with a given cell suspension the inhibition of the respiration

[2] We are indebted to Dr. G. Gomori for this information.

was independent of the glucose concentration over the range of 0.4 per cent to 1 per cent. Although of smaller magnitude, the rate of respiration in the presence of glucose was considerably better maintained than that in the absence of added substrate. If glucose was tipped into the main compartment after the cells had been allowed to respire 9 hours in the absence of added substrate, then the inhibition of oxygen uptake by glucose was no longer apparent.

No difference in the rate of respiration with or without sugar was observed when air was substituted for 100 per cent oxygen as the gas phase. Most respiration experiments were performed with air as the gas phase.

The endogenous respiration was unaffected by D-ribose (1 per cent) as well as by the following substances: L-proline (0.02 M), DL-lactate (0.02 M), L-aspartate (0.02 M), glycolate (0.02 M), and pyruvate (0.02 M). Proline, aspartate, and glycolate were also without influence on the endogenous respiration when tipped into the main compartment 9 hours after the beginning of the experiment. Octanoate (0.003 M) depressed the endogenous respiration about 10 per cent. Succinate (0.01 M) enhanced the endogenous respiration about 25 per cent.

NaCN (10^{-2} M) and NaN_3 (10^{-2} M) inhibited the endogenous respiration 96 per cent and 75 per cent, respectively, over an initial 120-minute period.

Changes in the rate of oxygen uptake induced by various dyes were markedly dependent on the presence of glucose in the suspension medium. In the absence of added glucose, 2,4-dinitrophenol (5×10^{-5} M) had no significant effect on the respiration, while progressive inhibition was caused by methylene blue (5×10^{-4} M) and Janus green 6B (5×10^{-5} M). When glucose was present, however, 2,4-dinitrophenol caused an initial acceleration of the rate of respiration by as much as 100 per cent, which was followed by a rapid decline of respiratory rate to practically nil. Under the same conditions, methylene blue accelerated the oxygen uptake, while Janus green 6B was inhibitory (although the inhibition was not so great as that seen in the absence of glucose, and only appeared after the first hour of the experiment).

It must be emphasized, however, that the influence of both glycolyzable sugars and dyestuffs on the endogenous respiration reported above are dependent upon the suspension medium employed. If the cells are washed with and added to the vessels in isotonic NaCl-KCl solutions, then the respiration of the cells in the presence of either tris-(hydroxymethyl)-aminomethane (0.01 M) or glycyl-glycine (0.01 M) buffer at pH 7.4 is markedly inhibited by 2,4-dinitrophenol, and the response to added glucose is often dependent upon the further presence of inorganic ortho-phosphate in the medium. A detailed study of factors influencing the endogenous respiration will be reported in a subsequent communication.

Respiratory quotient.—The depression of the endogenous respiration by glucose and fructose was accompanied by a corresponding increase in the respiratory quotient. Thus, the mean R.Q. for four determinations in the absence of glucose was 0.86, while in the presence of glucose the value rose to 1.16. DL-Lactate (0.02 M) affected neither the rate of respiration nor the R.Q. Assuming that the rate of respiration of these cells was not grossly affected by the presence of CO_2 (an assumption necessary for the calculation of the R.Q. by the method employed), it would appear that the presence of glycolyzable sugar elicits qualitative as well as quantitative changes in the respiration.

The Enzymic Activities of Tumor Cell Homogenates and Other Preparations

Hexokinase.—Differences in the rate of glycolysis of low concentrations of glucose and fructose by slices of various tumors have been interpreted in terms of the different affinity of tumor hexokinase for these two sugars (23). The higher rate of glycolysis of washed ascitic tumor cells in the presence of low concentrations of glucose as compared with fructose (*vide supra*) is also reflected in the behavior of the hexokinase of this tissue towards these compounds.

Chart 2 records the results of a typical experiment in which the hexokinase activity of an aqueous extract of an acetone powder of washed ascitic tumor cells was determined manometrically. When glucose and fructose were present at a final concentration of 0.016 M, the latter was phosphorylated at 123 per cent of the rate of glucose. However, glucose at a final concentration of 0.00066 M was utilized at more than 80 per cent of the rate of 0.016 M glucose until virtually all the sugar had been phosphorylated, at which time the rate fell to zero. Low concentrations of fructose were phosphorylated at much slower rates; thus, 0.0033 M fructose and 0.00066 M fructose were utilized at 33 per cent and 3 per cent of the rate of 0.016 M fructose, respectively. One curve in Chart 2 demonstrates that the ascitic tumor cell hexokinase, like that of yeast and normal and other malignant tissues, is a magnesium-activated protein; the small residual hexokinase activity observed in the absence of added magnesium is presumably a consequence of the presence of small amounts of this ion in the enzyme preparation.

Diphosphopyridine nucleotide breakdown.[3]—In the presence of glycyl-glycine buffer (pH. 7.4), the breakdown of DPN catalyzed by homogenates and fractions thereof of the ascitic tumor cells appeared to take place by a mechanism similar to that occurring in mammalian brain (20), the molecule being ruptured at the nicotinamide-ribose, rather than at the pyrophosphate (16) bond. The destruction of DPN, as measured with the aid of

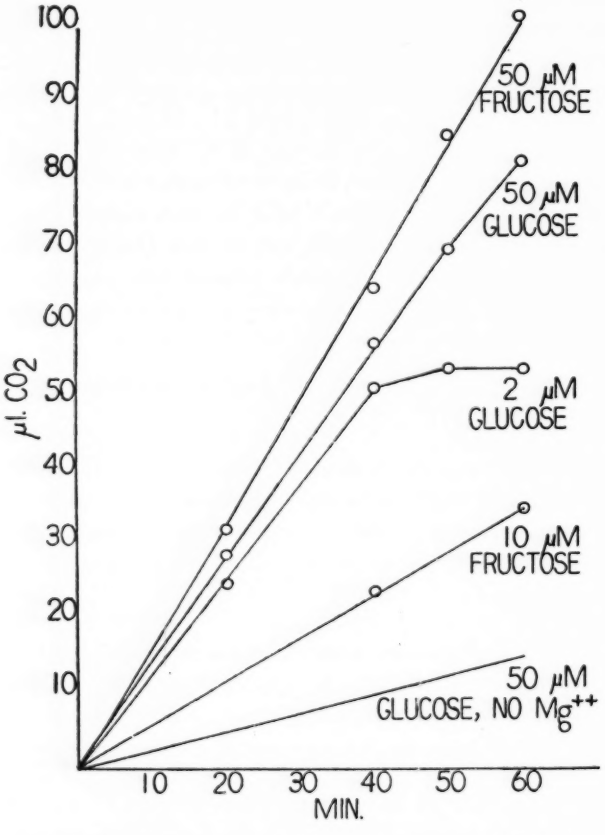

CHART 2.—Hexokinase activity of an aqueous extract of an acetone powder of ascitic tumor cells.

the alcohol dehydrogenase-ethanol system, was found to be equivalent, on a molar basis, to the formation of nicotinamide (determined chemically) and to the disappearance of the nicotinamide-riboside linkage (determined fluorimetrically). Furthermore, 0.01 M nicotinamide virtually abolished the breakdown of DPN, as well as the disappearance of the nicotinamide-riboside linkage. Fractionation of ascites tumor homogenates prepared in 0.25 M sucrose (8) showed that although DPN-ase activity was demonstrable in all the fractions studied, only the mitochondrial fraction exhibited a specific activity in excess of that of the unfractionated homogenate. A representative experiment is summarized in Table 1.

[3] These experiments were performed in conjunction with Dr. G. A. Burnett.

Phosphorylation coupled with the oxidation of succinate.—With the fortified medium described above it was found that, concomitant with the oxidation of succinate by washed ascitic tumor cytoplasmic particles isolated from homogenates prepared in either 0.15 M KCl or 0.25 M sucrose, a marked esterification of inorganic P occurred, the P/O ratio being greater than unity in some experiments. Omission of succinate from the suspension medium reduced the oxygen uptake to negligible amounts and abolished the phosphate disappearance (in some cases a small output of inorganic P was observed). Table 2 depicts the results of two typical experiments.

Attempts to demonstrate octanoate oxidation by washed cytoplasmic particles.—A few experiments were performed in which ascitic tumor cell mitochondria, washed twice with 0.25 M sucrose, were added to Warburg flasks containing the following components in a final volume of 3.0 ml.: 30 µM ortho-phosphate of pH 7.4; 15 µM $MgCl_2$; 0.11 µM cytochrome c; 50 µM nicotinamide; 2.3 µM DPN;

TABLE 1

DPN-ASE ACTIVITY OF VARIOUS FRACTIONS OF HOMOGENATE OF ASCITIC CELLS

FRACTION	SPECIFIC ACTIVITY (µM DISAPPEARANCE OR FORMATION/MG N/HR) Δ Nicotinamide	Δ DPN
Whole homogenate	0.24	0.24
Nuclei	0.14	0.15
Mitochondria	3.55	4.70
"Supernatant"*	0.24	0.11

* This fraction was not further separated into microsomes and soluble fractions.

TABLE 2

PHOSPHORYLATION COUPLED TO SUCCINATE OXIDATION CATALYZED BY CYTOPLASMIC PARTICLES ISOLATED FROM ASCITIC CELLS

Exp.*	Substrate	Δ P (µM)	Δ O_2 (microatoms)	P/O
1	Succinate	− 6.4	5.4	1.18
2	None	+ 0.7	0.8	
	Succinate	−16.1	14.0	1.15

* Exp. 1.—Particles isolated from homogenate of ascitic cells prepared in 0.15 M KCl. The preparation was washed twice with and finally suspended in this medium; 10 µM inorganic ortho-phosphate added to reaction mixture; duration of experiment, 16 min.

Exp. 2.—Particles isolated from homogenate of ascitic cells prepared in 0.25 M sucrose. Preparation washed once with and finally suspended in this medium; 20 µM ortho-phosphate added to reaction mixture; duration of experiment, 60 min.

6 µM ATP; and 150 µM KCl. Negligible oxygen uptake occurred under these conditions whether or not 3 µM of octanoate was added. The addition of 100 µM succinate gave rise to an oxygen consumption of 9–10 microatoms O_2/45 min., 10 µM succinate being oxidized at about 40 per cent of this

rate. The further addition of 3 μM octanoate to a system containing either 3 μM or 10 μM succinate did not enhance the oxygen uptake over that of the blanks containing succinate alone; the octanoate actually depressed the rate of succinate oxidation by about 25 per cent. Similarly, the addition of 15 μM oxalacetate gave rise to an oxygen consumption which was not significantly different from that in the absence of added substrate, and no increased oxygen consumption occurred when flasks were set up containing, in addition to the suspension medium, both 15 μM oxalacetate and 3 μM octanoate.

Activity of other enzyme systems in homogenates of ascitic tumor cells.—The succinoxidase and malic dehydrogenase activities of homogenates of these cells prepared in 0.15 M KCl were found to be 17.4 and 15.8 μl O_2/mg dry weight/hour, respectively.

High rates of anaerobic glycolysis were found to occur when hexose diphosphate was added to homogenates prepared in isotonic KCl in the presence of Ng^{++} ions, DPN, nicotinamide, fluoride, pyruvate, adenosine monophosphate, and inorganic orthophosphate or arsenate. This triose phosphate-pyruvate oxido-reduction could also be linked with molecular oxygen on the further addition of either cytochrome c or brilliant cresyl blue in the absence of pyruvate in the fluoride-containing medium. When pyruvate was used as an electron acceptor, the further addition of yeast hexokinase and fructose gave rise to a marked esterification of inorganic P.

The Composition and Enzymic Properties of the Ascitic Plasma

The protein content of nine different pooled samples varied from 2.1 per cent to 3.3 per cent, as determined spectrophotometrically. The nonprotein nitrogen content of the samples varied from 21 mg/100 ml to 24 mg/100 ml, i.e., approximately 5 per cent of the total N. Calculation of the protein content of the ascitic plasma from the differences between the total N and nonprotein nitrogen gave values in excellent agreement with those obtained from the spectrophotometric data.

An electrophoretic examination of the proteins of the ascitic plasma was kindly undertaken by Mrs. Anne Cleveland of this laboratory. Pooled ascitic fluid from the peritoneal cavities of CF1 male mice (inoculated 7 days previously) was centrifuged at 2,000×*g* to remove all cellular material. The fluid was dialyzed for 5 days against veronal buffer of pH 8.6 and ionic strength 0.1, and was then subjected to electrophoresis. Computation of the percentage content of the various protein fractions gave the values shown in Table 3.

Ultraviolet absorption spectra, measured over the range of 240 mμ to 300 mμ, showed distinct peaks between 275 mμ and 285 mμ and no peak at 260 mμ. In the visible range an absorption maximum at 410–415 mμ was usually observed, the magnitude of which varied considerably from sample to sample.

Enzyme activities.—

1. Hydrolytic enzymes: Table 4 gives the values obtained for the activity of a number of enzymes present in the ascitic plasma. The estimations were carried out under conditions where the activity was proportional to the amount of ascitic plasma added and to the time of incubation. The acid phosphatase activity was much greater than the practically negligible alkaline phosphatase activity. The choline esterase activity appeared to be mainly of the 'pseudo' choline esterase type, since the same volume of plasma which catalyzed a vigorous breakdown of acetylcholine brought about a very slight hydrolysis of acetyl-β-methylcholine. The addition of $MgCl_2$ (0.004 M) did not significantly affect the rate of acetylcholine hydrolysis. No significant DPN-ase activity was ex-

TABLE 3

ELECTROPHORETIC ANALYSIS OF THE PROTEINS OF THE ASCITIC PLASMA

Component	Per cent content calculated from: Descending peak	Ascending peak
Albumin	57.8	58.0
α_1-globulin	10.8	11.5
α_2-globulin	7.6	7.3
β-globulin	20.7	21.3
γ-globulin	3.7	3.3

TABLE 4

ENZYMIC ACTIVITIES OF ASCITIC PLASMA

Enzyme	Vol. of ascitic plasma used for determination (ml.)	Protein content of ascitic plasma used* (per cent)	Activity of enzyme
Acid Phosphatase	0.5	2.73	84.0†
	0.5	2.21	72.0†
Alkaline Phosphatase	0.5	2.73	3.0†
	0.5	2.21	6.0†
β-glucuronidase	0.5	2.73	20.4‡
	0.5	2.21	12.0‡
Choline esterase§	0.2	2.21	2055.0‖
'True' choline esterase#	0.4	2.21	73.0‖

* Determined spectrophotometrically.
† μg. phenol liberated per 1 ml. ascitic plasma per hour.
‡ μg. phenolphthalein liberated per 1 ml. ascitic plasma per hour.
§ Acetylcholine as substrate.
‖ Acetyl-β-methylcholine as substrate.
μl. CO_2 evolved per 1 ml. ascitic plasma per hour.

hibited by 0.5 ml. ascitic plasma, which possessed considerable β-glucuronidase activity.

2. Glycolytic enzymes: Estimations of the aldolase activity of the ascitic plasma showed that 1.0 ml. split approximately 3.5 μM HDP per hour and hence should give rise to approximately 7 μM triose phosphate per hour. These values are equivalent to about 80 aldolase units of Sibley and Lehninger (28). One ml. of the ascitic plasma catalyzed the formation of 8.9 μM phosphopyruvate from 3-phosphoglycerate in 1 hour at 37°.

The relatively high activity of these two glycolytic enzymes suggested that the ascitic plasma might possess the ability to glycolyze HDP. This possibility was examined by two methods of approach. A series of Warburg manometers were set up containing in a final volume of 2.7 ml. in the main compartment: 13.5 μM HDP; 120 μM NaF; 40 μM Na pyruvate; 10 μM Na arsenate; 150 μM $NaHCO_3$, while the side-arm contained 0.3 ml. of a solution containing 6.5 μM DPN. After the plasma samples were placed in the flasks, the latter were attached to the manometers and gassed with 95 per cent N_2-5 per cent CO_2. After 13 minutes of equilibration at 37°, the DPN was tipped into the main compartment from the side-arm and the gas exchange measured over the next hour. Blanks containing all the reagents except the HDP were set up to correct for any gas exchange on tipping the DPN into the main compartment. Duplicate vessels containing 1.0 ml. and 0.5 ml. of ascitic plasma (2.4 per cent protein) gave rise to the evolution of 0.50 and 0.08 μM CO_2, respectively, in 1 hour, while the blanks without HDP evolved 0.31 μM CO_2 during this period. It is evident that no significant triose phosphate-pyruvate oxido-reduction occurred under these conditions. Attempts were also made to study the possible reduction of DPN when HDP and the ascitic plasma were allowed to react together. Beckmann spectrophotometer cuvettes were set up containing the following constitutents in a final volume of 3.00 ml.: 40 μM glycyl-glycine buffer of pH 7.5, 10 μM Na arsenate, 120 μM NaF, 0.4 μM DPN, and 1.0 ml. of ascitic plasma. The reaction was initiated by the addition of 13.5 μM HDP, the blank cells containing water in place of this substrate. The absorption of the reaction fluid at 340 mμ was then observed at 22°. No increase in absorption was observed over a period of 1 hour. It may be tentatively concluded from these experiments that triose phosphate deydrogenase is virtually absent from the ascitic plasma.

SUMMARY

Washed Ehrlich ascites tumor cells exhibited high rates of anaerobic and aerobic glycolysis which were dependent upon the addition of either glucose or fructose. Considerable oxygen uptake was observed when these cells were suspended in the absence of added substrate, and the endogenous respiration was suppressed on the addition of glycolyzable sugar, the respiratory quotient being elevated at the same time. The activity of a number of enzymes in homogenates of the tumor cells was studied, and mitochondria isolated from such preparations were shown to catalyze a marked uptake of inorganic phosphate concomitant with the oxidation of succinate. The rate of phosphorylation of various concentrations of glucose and fructose by the hexokinase present in aqueous extracts of acetone powders of the tumor was shown to parallel the rate of anaerobic glycolysis of these sugars by the intact tumor cells.

The ascitic plasma was found to contain a number of hydrolytic and glycolytic enzymes but did not possess the ability to glycolyze hexose diphosphate to lactate.

ACKNOWLEDGMENTS

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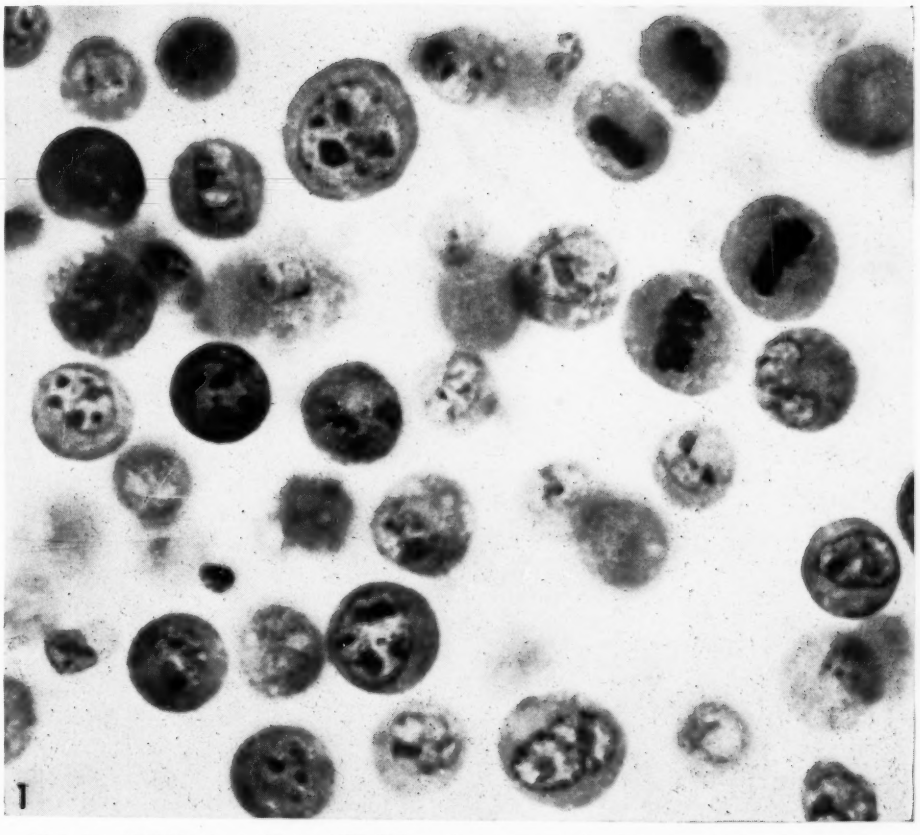

FIG. 1.—Cell block sections of Ehrlich ascites tumor cells. Fixed in Zenker-formalin; imbedded in celloidin and stained with hematoxylin-eosin-azure. Mag. × 1,000.

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The Concentration of Folic Acid in Leukocytes

Observations on Normal Subjects and Persons with Leukemia*

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Daft and Sebrell (9) in 1943 presented evidence that folic acid is essential for leukocyte maturation in the rat, and they thus established this vitamin as the first nutrient factor known to influence leukocyte production in a decisive manner. These observations have been abundantly confirmed and extended to include a variety of animal species (2, 8, 11).

Since leukemia is characterized by excessive proliferation of leukocytes and their precursors, it seemed pertinent to determine the amount of folic acid present in the leukocytes of persons with various types of leukemia and to compare such values with those obtained in normal subjects. A preliminary report (3)[1] of the results showed that the folic acid content of the leukocytes is higher in leukemic than in normal subjects, and this is particularly true of the more acute forms of the disease. The detailed data of the study will be given at this time. In addition, since metabolic investigations have shown the importance of folic acid conjugate cleavage (4, 21), measurements of the ratio of free to bound folic acid, as well as estimations of the enzyme folic acid conjugase contained in the leukocytes, have been made in a number of cases.

METHODS AND CLINICAL MATERIAL

Patients are classified according to type of leukemia based on peripheral blood and bone marrow examinations, but detailed clinical reports are not included. At the time of study, except where it is specifically stated, they either had never received therapy or had received no recent therapy. Normal subjects were laboratory workers and medical students.

Venous blood samples were obtained when the subjects were in a fasting state, and the method of Gorham and co-workers (13) was employed to separate the leukocytes and to prepare them for analysis. As pointed out by these investigators, this fraction also contains some of the blood platelets. The amount used for folic acid analysis was measured on a volume rather than on a weight basis and is expressed as cubic centimeters of leukocytes obtained by centrifugation to a state of maximum packing.

With the realization that the greater portion of the folic acid present in animal tissues is bound in the form of a conjugate not directly utilizable by micro-organisms of the assay procedures (5), it became necessary to investigate methods for conversion of the folic acid present in the leukocytes to the free state. It was found that the leukocytes themselves contained a folic acid conjugase with a pH optimum of 4.5. Determination of the concentration of this enzyme according to the method of Bird and associates (5) showed that it was present in sufficient quantities to effect complete conjugate cleavage. Accordingly, a standard procedure was adopted whereby the centrifuged leukocytes were diluted 50 times with equal quantities of distilled water and 1 per cent sodium acetate buffer of pH 4.5 and incubated at 37° C. for an 18-hour period. After heat inactivation, the leukocyte dilutions were adjusted to the appropriate pH and assayed microbiologically using either *L. casei* or *S. faecalis* as the test organism (14, 20). In these assays, synthetic pteroylglutamic acid was used to prepare the standard curve. The addition of hog kidney conjugase (5) gave no increase in leukocyte folic acid values following this treatment. In some instances the free folic acid of the leukocyte was also determined, and here the leukocytes

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[1] We wish to correct an error in this abstract. The values for folic acid should be recorded as millimicrograms rather than as micrograms.

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were assayed directly without incubation at pH 4.5.

EXPERIMENTAL RESULTS

In Table 1, values are recorded for the amount of folic acid conjugase present in the leukocytes of three normal subjects and three patients with leukemia. Expressed as conjugase units per cubic centimeter of leukocytes, the values obtained in leukemia are similar to those of normal subjects. As will be seen from subsequent tables where leukocyte folic acid concentrations are shown, the amount of enzyme is more than sufficient under the experimental conditions employed to convert all the folic acid of the leukocytes to the free state.

TABLE 1

FOLIC ACID CONJUGASE CONTENT OF LEUKOCYTES

Subject	Conjugase units*/cc leukocytes
Normal	100
"	130
"	88
Leukemia	190
"	110
"	120

* One unit represents the amount of enzyme necessary to release 1 μg. folic acid from crystalline hexaglutamyl conjugate in 1 hour.

TABLE 2

THE PARTITION OF FOLIC ACID IN LEUKOCYTES

Subject	Total (mμg/cc)	Free (mμg/cc)	Per cent free
Normal	160	13	8
"	130	23	18
"	80	17	21
Leukemia	360	37	10
"	350	64	18
"	240	28	12
"	260	17	7
"	220	40	18

By assaying the folic acid content of leukocytes before and after digestion at pH 4.5, the free and total values were obtained (Table 2) for three normal subjects and five patients with leukemia. The free folic acid varies from 8 to 21 per cent of the total amount of vitamin present, and, since the enzymic activity of folic acid conjugase is very slight at the natural pH of the leukocyte, these values would seem to approximate the *in vivo* status as regards the degree of folic acid conjugation. Similar ratios of free to total folic acid pertain in normal subjects and leukemia patients.

The leukocytes of twelve normal subjects were assayed for folic acid, and the values obtained are shown in Table 3. They range from 40 to 180 mμg. per cubic centimeter of packed leukocytes with an average of 80 mμg. These values are to be compared with those in Table 4 where the folic acid content of leukocytes from patients with various types of leukemia are recorded. All the patients were in the leukemic rather than the subleukemic or aleukemic phase of their disease. Considered as a whole, although a wide variation occurs in the vitamin content of these leukocytes, the values tend to be increased above those obtained with leukocytes from normal individuals. When the leukemic patients are classified according to the type of the disease they manifest, the leukocyte folic acid values become more consistent. Thus, for five cases of chronic lymphocytic leukemia, the range is from 70 to 160 mμg/cc of

TABLE 3

FOLIC ACID IN LEUKOCYTES OF NORMAL PERSONS

Subject	Folic acid (mμg/cc)	Subject	Folic acid (mμg/cc)
PW	45	MV	130
HA	75	MS	65
BB	40	MT	100
MJ	60	MB	85
RH	180	CM	75
MC	50	JJ	60
			Av. 80

TABLE 4

FOLIC ACID IN LEUKOCYTES OF PATIENTS WITH LEUKEMIA

Subject	Type of leukemia	Folic acid (mμg/cc)
CR	Chronic lymphocytic	160
TA	" "	70
MR	" "	90
BE	" "	90
RE	" "	150
		Av. 120
MA	Lymphosarcoma	85
FA	"	250
RE	"	420
		Av. 250
PA	Chronic granulocytic	150
HI	" "	180
HO	" "	220
HA	" "	75
WH	" "	85
WA	" "	160
NO	" "	125
MO	" "	260
		Av. 155
GR	Acute granulocytic	270
DA	" "	560
CO	" "	800
MC	" "	390
RO	" "	460
ST	" "	350
LE	" "	400
GO	" "	415
		Av. 455

leukocytes with an average of 120 mμg. and for eight cases of chronic granulocytic leukemia, leukocyte folic acid values vary from 75 to 260 mμg/cc of leukocytes, with an average of 155 mμg. When eight cases of acute granulocytic leukemia are considered as a unit, a range of 270–800 mμg. of folic acid/cc of leukocytes is found, with an average of 455 mμg. This acute form of the disease, characterized by a preponderance of blast cells in the circulating blood and marrow, therefore shows a very significant increase in leukocyte folic acid concentrations over the chronic types, and the mean value is 5 times that of the average obtained in leukocytes of normal subjects.

Table 5 shows the effects of radiation treatment

TABLE 5

EFFECT OF RADIATION THERAPY ON FOLIC ACID CONTENT OF LEUKOCYTES OF LEUKEMIA PATIENTS

Subject	Folic acid content of leukocytes (mμg/cc)	
	Before therapy	After therapy
PA	150	80
ME	90	75
HI	220	60
HA	70	65

on the leukocyte folic acid values. In each of four cases, the vitamin content is reduced after therapy to a level which approximates the average of the normal subject series.

Subsequent to the completion of these studies, the presence in natural substances of a folic acid analog termed the citrovorum factor (folinic acid) was recognized (17), and with the isolation and synthesis of this compound (7, 18) the differentiation of folic acid and citrovorum factor activities was made possible (19). It has been found that most, if not all, the folic acid present in leukocytes of normal persons and patients with leukemia appears to be in the form of citrovorum factor. These observations are based on a differential microbiological assay procedure whereby leukocytes are assayed with *L. citrovorum* as well as with *S. faecalis* with synthetic citrovorum factor (folinic acid)[2] as the standard curve. The results are shown in Table 6. The assay values for *L. citrovorum*, which responds only to folinic acid, are nearly identical with the assay values for *S. faecalis*, which responds to both folic and folinic acids, indicating the predominance of the folinic acid analog in the leukocytes.

DISCUSSION

In the following discussion and summary the term folic acid is intended to include folinic acid.

[2] We are indebted to Dr. William Shive of the University of Texas for this preparation.

Since evidence obtained from folic acid conjugate studies in pernicious anemia patients (4, 21) and in *in vitro* tyrosine oxidation processes (16) indicates that in order for folic acid to be metabolically available it must be present in the free form, the amounts of free folic acid and folic acid conjugase assume significance in leukocyte folic acid economy. In accordance with results of assays on other animal tissues (5), from 80–90 per cent of the folic acid content of leukocytes was found to be bound in the form of a conjugate. However, a very high concentration of folic acid conjugase was also observed to be present. Since similar amounts of free folic acid and folic acid conjugase activity were found in leukocytes of both leukemia patients and normals, there appears to be no basis for the possible assumption that, in leukemia, the folic acid of the leukocyte is not metabolically available.

The leukocytes of leukemia patients have also been assayed for other vitamins, notably thiamine (1) and ascorbic acid (18). Here again, higher concentrations have been found than in normal subjects, but there was no correlation between vitamin concentration and cell immaturity. In this

TABLE 6

FOLID ACID CONTENT OF LEUKOCYTES EXPRESSED AS FOLINIC ACID

Subject	Assay by *S. faecalis* (mμg folinic acid/cc)	Assay by *L. citrovorum* (mμg folinic acid/cc)
Normal	45	52
"	40	32
Leukemia	85	80
"	295	280
"	100	110

study such a correlation has been shown by comparison of values obtained in acute and chronic forms of the disease. Since the effects of therapeutic irradiation in leukemia are manifested chiefly by a diminution in the rate of proliferation of leukocyte precursors, the tendency for leukocyte folic acid concentrations to decrease following radiation therapy may be taken as further evidence of this correlation.

Pollack and co-workers (15) have found higher amounts of folic acid in various types of neoplastic tissue than in the corresponding normal tissue. It therefore appears that increased concentrations of folic acid may be a general characteristic of younger and rapidly growing cells.

One explanation for the great increase in folic acid concentration in the leukocytes of the acute leukemias, where values average approximately 5 times those of normal cells, is that the metabolic requirement for folic acid is greater in these leu-

kemic cells. In this connection, the action of folic acid antagonists is of extreme interest. Clinical evidence is in agreement that anti-folic acid compounds may be of therapeutic value in the treatment of the acute leukemias but are of little benefit in the more chronic forms of the disease (10, 12). If the younger and rapidly proliferating leukocytes concentrate folic acid to fulfill their relatively high requirements, they would be more susceptible to the action of folic acid antagonists. This explanation provides a rational basis for the use of the folic acid antagonists in the treatment of the acute leukemias.

SUMMARY

Values for the folic acid content, the percentage of free folic acid, and the folic acid conjugase activity of leukocytes in leukemia patients and normal subjects have been compared. Neither the folic acid conjugase activity nor the percentage of free folic acid showed significant variations under conditions of the experiment. However, the folic acid content was greater in leukemia, and the increase was correlated with the degree of cell immaturity, the highest leukocyte folic acid values being found in the acute forms of the disease. As a result of x-ray therapy, leukocyte folic acid values tended to be reduced. The predominant form of folic acid in the leukocyte has been shown to be folinic acid.

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Nitrogen Mustard Inhibition of Azo Dye Carcinogenesis*

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Cellular growth and development may be altered appreciably by administration of the nitrogen mustards. The chemotherapeutic value of these compounds in the treatment of leukemia and other neoplasms has been well established (5). In addition, the studies reported by Auerbach, Tatum, Karnofsky, and others have indicated that these agents are mutagenic for Drosophila (1, 2), Neurospora (21), bacteria (20), and chick embryonic tissue (13). Recent reports from this and other laboratories provide conclusive proof that the radiomimetic nitrogen mustards are active carcinogens (3, 10, 12).

Berenblum (4) demonstrated that the addition of mustard gas in a 1 per cent concentration inhibited the production of warts by tar. Further studies established that the inhibition was a direct effect of the chemical on the animal. The carcinogen, 1,2,5,6-dibenzanthracene, gave a similar effect. Recently, Riegel and associates (19) have shown that administration of 1,2,5,6-dibenzofluorene reduced the tumor incidence resulting from methylcholanthrene. In a somewhat comparable study, Richardson and Cunningham (18) found that the application of methylcholanthrene resulted in a lowering of the tumor incidence in rats fed diets containing 3′-methyl-4-dimethylaminoazobenzene (3′MeDAB). There have been several instances, however, when the administration of one carcinogenic agent did not alter the action of a second carcinogenic agent (8). The incidence of ear tumors in Swiss mice exposed to ultraviolet light was not altered by weekly injections of nitrogen mustard.[1]

Since the nitrogen mustards are capable of inhibiting cellular development and also of inducing somatic mutations and tumors, it appeared worthwhile to study the effect of these agents on the process of cancer induction by other compounds. An alteration of this process by the nitrogen mustard may provide information bearing on the mechanism of carcinogenesis. In the present study, methylbis(β-chloroethyl)amine (HN2) was injected subcutaneously into albino rats fed diets containing 3′MeDAB. The effect of this treatment on the resulting incidence of liver tumors is reported.

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[1] Unpublished data from this laboratory.

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METHODS

Male albino rats of the Holtzman Sprague-Dawley strain were maintained on a purified diet (9) containing 0.06 per cent 3′MeDAB until tumors were evident by palpation or by laparotomy of control animals. At this time, the rats were placed on a purified basal diet containing no dye for an additional 4–5 weeks to allow the tumors to grow to recognizable size (15). The animals treated with nitrogen mustard received subcutaneous injections once weekly of 0.5 mg HN2/kg of body weight; the drug was administered as a 0.01 per cent solution of the hydrochloride salt in physiological saline. All animals were weighed and food intakes for the various groups were determined throughout the study. The food intake was lower with the 3′MeDAB diet than with the basal diet alone. Animals fed the dye-containing diet, however, maintained or gained weight slowly. Administration of HN2 to dye-fed rats did not appreciably change the food intake or the maintenance of body weight.

Initially, two series of experiments were carried out. The first series, conducted over a 10-week period, comprised three groups: 3′MeDAB controls fed the dye as described above; animals fed the dye and treated weekly with HN2; and HN2 controls fed basal diet and similarly treated. The second series was a repetition of the first and also included groups to show the effect of varying the injection period of HN2 during the feeding of the carcinogen. This series included: 3′MeDAB controls fed the dye for 12 weeks, animals treated with HN2 the first 2 weeks of a 10-week dye-feeding period, rats treated with HN2 the last 4 weeks of the feeding period (sixth to tenth week on experiment), animals treated weekly during a dye-feeding period of 10 weeks, animals treated throughout a 12-week feeding period, and control animals

maintained on the dye-free diet and treated with HN2 for 10 weeks. A third confirmatory series included: rats maintained on this diet and treated with HN2 the first and second week, and animals fed the dye and treated weekly during the 12-week period.

At the end of 15–16 weeks, when control animals fed the diets containing 3′MeDAB showed a high percentage of palpable tumors, the rats were sacrificed by decapitation and exsanguination. At this time, gross observations of the condition of livers were made, and each liver was designated as being tumorous, probably tumorous (containing areas resembling tumor tissue and severely cirrhotic), cirrhotic, or normal. While the distinction between these groups was slight, all were judged and reported by the same standard so that results are valid. Where chemical determinations of nucleic acids were to be made, the livers were perfused *in situ* with cold saline, removed, and stored at 0° C. for several hours. The liver tissues were homogenized; phosphorus, nitrogen, desoxyribonucleic acid and ribonucleic acid were determined as described in a previous publication (11).

RESULTS

The tumor incidence in animals fed the carcinogenic azo dye 3′MeDAB and treated with nitrogen mustard, as compared to control dye-fed animals, is summarized in Table 1. In determining the percentage of tumor formation, the "probable" tumors were counted as tumors. Cirrhotic livers were considered nontumorous unless definite tumorous lesions were evident.

Control animals fed the diet containing 3′MeDAB for 10 or 12 weeks showed a 90–100 per cent incidence of liver tumors (Table 1). Both untreated and nitrogen mustard-treated control animals maintained on the basal diet alone had normal livers. The greatest inhibition of tumor development was found in animals fed the dye for 10 weeks and treated once weekly with HN2 throughout the feeding period. In this group, less than one-third of the animals exhibited tumors, and most of the remaining rats had livers of normal appearance.

An initial observation was made on one rat in the first series of experiments which, because of inflammatory lesions at the subcutaneous area, was injected with HN2 the first 3 weeks of the experiment only. After 10 weeks on the 3′MeDAB diet, the liver of this animal was normal; this indicates that inhibition occurs primarily during the first few weeks. Subsequent studies partially confirmed this initial observation. The incidence of tumors was found to be 43 per cent when HN2 was administered the first 2 weeks only. Similar results were obtained when the dye-containing diet was fed for 12 instead of 10 weeks. In the 12-week series the control animals had a 92 per cent incidence of liver tumors, while animals treated weekly with HN2 showed an incidence of 57 per cent. In the group wherein the nitrogen mustard was injected in the first 2 weeks only, the tumor incidence was 63 per cent (Table 1). The nitrogen mustard did not inhibit liver tumor formation when administered during the latter weeks of the azo dye feeding. This may be seen from the 100 per cent tumor

TABLE 1

THE REDUCTION OF TUMOR INCIDENCE BY HN2 IN RATS FED DIETS CONTAINING 3′METHYL-4-DIMETHYLAMINOAZOBENZENE

Diet	No. weeks	Treatment	Description of liver at termination of experiment	Survival at end of dye-feeding period	Survivors without tumors	Tumor incidence*
Basal	15	HN2†, 10 wks.	All livers normal	9/9	9	0/9
3′MeDAB‡	10	None	Tumors present in all livers. Most of surrounding tissue cirrhotic.	9/9	0	9/9
Basal	6					
3′MeDAB	10	HN2, first 2 weeks	One with definite tumor, two probable. Four livers were nearly normal.	7/8	4	3/7
Basal	6					
3′MeDAB	10	HN2, 10 wks.	Tumors present in two livers. Others normal or nearly normal.	7/9	5	2/7
Basal	6					
3′MeDAB	10	HN2, last 4 weeks	Tumors in all livers. Livers enlarged and severely cirrhotic.	7/8	0	7/7
Basal	6					
3′MeDAB	12	None	One normal, tumors present in all others. Livers enlarged, cirrhotic.	12/14	1	11/12
Basal	4					
3′MeDAB	12	HN2, first 2 weeks	Five small tumors. Most livers were cirrhotic.	8/8	3	5/8
Basal	4					
3′MeDAB	12	HN2 ,12 wks.	Tumors in eight livers. Most livers were somewhat enlarged and cirrhotic.	14/15	6	8/14
Basal	4					

* Tumor incidence: number with tumors over number surviving at end of dye-feeding period.
† HN2: methylbis(β-chloroethyl)amine hydrochloride. Administered subcutaneously, 0.5 mg/kg body weight.
‡ 3′MeDAB: 3′methyl-4-dimethylaminoazobenzene. Incorporated in basal diet at a level of 0.06 per cent.

incidence obtained when HN2 was injected the last 4 weeks prior to the basal diet period. In addition, treatment with HN2 of several rats bearing small liver tumors did not appreciably modify the growth or development of the tumors.

From the data presented in Table 1, it is evident that administration of HN2 does not completely inhibit liver tumor formation. Also, it may be noted that a higher incidence of tumors was obtained when the azo dye and HN2 were administered for 12 weeks instead of 10 weeks. Thus, prolonged feeding of the dye might partially overcome the inhibiting effect of HN2. Several animals from a group fed the diet containing 3′MeDAB and also a group fed this same regimen but treated with HN2 were sacrificed at 7 weeks so that the condition of the liver could be observed. In the former control group, seven of the eight livers were cirrhotic, and one tumor was present. In the latter group of eight animals, three livers were cirrhotic and five appeared to be completely normal. Thus, the observation at 7 weeks confirms the results obtained at the termination of the experiments as shown in Table 1.

From data obtained in the present study it may be noted that the livers of animals fed a similar diet but treated with HN2 showed a liver concentration of DNA of 300–400 mg. per cent. This is indicative of early precancerous stages in normal animals fed the dye only. Ribonucleic acid (RNA) values proved to be inconsistent, and little significance can be attached to them. Values intermediate between normal liver and tumor levels were obtained in the livers of animals treated with nitrogen mustard. The nitrogen concentration of the various tissues remained essentially constant; however, the level in the livers of animals fed the diet containing the azo dye and treated with HN2 for 7 weeks was somewhat lower than that in the liver tissues of other groups (Table 2).

TABLE 2

THE EFFECT OF HN2 ON THE NUCLEIC ACID COMPOSITION OF LIVERS OF RATS FED DIETS CONTAINING AZO DYE

Diet	No. weeks	Treatment	Description of liver	Desoxyribonucleic acid (mg. per cent)	Ribonucleic acid (as ribose) (mg. per cent)	Nitrogen (mg. per cent)
Basal	7	None	Normal	266	184	2,343
3′MeDAB*	7	None	Cirrhotic	422	146	2,077
3′MeDAB	7	HN2†, 7 wks.	Normal	379	144	1,845
Basal	15	HN2, 10 wks.	Normal	308	143	2,420
3′MeDAB	10					
Basal	5	None	Tumorous	600	168	2,490
3′MeDAB	12					
Basal	4	HN2, 12 wks.	Cirrhotic	393	135	2,285
3′MeDAB	10	HN2, first				
Basal	5	2 weeks	Normal	396	158	2,415
3′MeDAB	10					
Basal	6	HN2, 10 wks.	Normal	358	100	

* 3′MeDAB: 3′methyl-4-dimethylaminoazobenzene. Incorporated in basal diet at a level of 0.06 per cent.
† HN2: methylbis(β-chloroethyl)amine hydrochloride. Administered subcutaneously, 0.5 mg/kg body wt.
Each group was composed of tissue from three to seven animals.

Chemical studies of livers obtained on sacrifice of the animals are given in Table 2. Livers used for analysis were chosen as representative of each group; the general classification is given in the table. The desoxyribonucleic acid (DNA) in normal rat liver is approximately 250–275 mg. per cent, while in liver tumors induced by 3′MeDAB the average values range from 600 to 700 mg. per cent. In a previous study (9), it was found that feeding of diets containing this azo dye resulted in a progressive increase in the liver DNA, until a precancerous level of 506 mg. per cent was reached.

DISCUSSION

The induction of liver tumors by the carcinogenic azo dye, 3′MeDAB, was inhibited by the simultaneous administration of the nitrogen mustard, HN2. This was predicted from the nucleotoxic properties of the mustard. The mechanism of hepatoma formation may possibly be attributed to mitotic stimulation in the liver tissues in response to the azo dye. An increase in mitotic activity is evident from the histological and chemical studies of Price and associates (16, 17) and of Cunningham *et al.* (6, 7). An increase in the numbers of nuclei and a corresponding increase in DNA per gram of liver tissue was noted as the carcinogenic azo dyes were administered. The increase in DNA in the liver tumors produced is further evidence for this proposed mechanism of tumor formation, i.e., mitosis accompanied by or following upon increased synthesis of this nucleic acid. Conversely, studies with nitrogen mustard have shown

that this chemical causes a marked depression of DNA synthesis and an inhibition of mitosis. Results of the experiments in the present study, with regard to both tumor incidence and DNA concentrations, are evidence for the antagonistic effect of HN2 on the azo dye, and can be interpreted to further support the proposed mechanisms of action of these two compounds.

Since the azo dyes stimulate mitosis in liver tissues and also bring about an increase in the concentration of liver DNA, it might be postulated that nuclei isolated from the livers of animals injected with P^{32} would contain relatively large quantities of the isotope. Studies completed in this laboratory (11) have shown that, following P^{32} administration, the specific activity of phosphorus was from 5 to 10 times greater in the liver DNA of animals fed diets containing 3'MeDAB than in normal control tissues. Recently, we have demonstrated that HN2 administered subcutaneously to animals fed the azo dye greatly reduced this incorporation of P^{32} into the nuclear DNA.[2] These observations are in agreement with the present findings and provide further evidence of the suppression of mitotic activity by the nitrogen mustard.

Two relationships between the dye-feeding period and nitrogen mustard injection were established. First, the nitrogen mustard apparently inhibits tumor formation only in the initial stage of azo dye administration; therefore, its preventive effect is exerted only in the beginning proliferative stage of tumor development. The results are in accord with experiments by Marshak on the effect of mustard gas (HS) on mitosis and P^{32} uptake in regenerating rat liver (14). He found mitosis to be greatly depressed when administration of HS immediately followed partial hepatectomy; inhibition of a lesser degree occurred if the treatment was initiated several hours after regeneration had begun. This suggested that if the mustard reaches the cells before mitosis begins, it prevents mitosis. Second, Marshak observed a quantitative relation between inhibition and the amount of mustard gas used, the inhibition decreasing with decreasing amounts of mustard gas. Studies reported here show a similar relationship between the amount of dye fed and the amount of nitrogen mustard injected, as was indicated by the higher tumor incidence in the 12-week feeding period.

This study provides further information concerning the mechanism of action of the nitrogen mustards. Thus, HN2 has been found to exert an inhibiting effect on the induction of tumors by a carcinogenic azo compound. The proposed mechanism involves an inhibition of mitosis caused in turn by a decreased synthesis of desoxyribonucleic acid in the individual cell.

[2] D. N. Ward, E. L. Brandt, and A. C. Griffin. The Effects of Nitrogen Mustard and a Pyridinium Compound on the Phosphorus^{32} Turnover in Rat Liver during Azo Dye Cancer Induction (manuscript submitted for publication).

SUMMARY

1. Albino rats were fed diets containing the carcinogenic azo dye, 3'methyl-4-dimethylaminoazobenzene, and also given subcutaneous injections of methylbis(β-chloroethyl)amine (HN2) at weekly intervals.

2. Animals fed the diets containing the dye exhibited a 90–100 per cent incidence of liver tumors after 10–12 weeks. Animals fed the same diet but treated with HN2 showed a tumor incidence of 30–60 per cent.

3. This inhibition of azo dye carcinogenesis by HN2 was most effective if the mustard was administered in the initial stages of the dye-feeding period. Injection of HN2 during the last 4 weeks did not affect the tumor incidence.

4. From chemical studies of the liver tissue, evidence was obtained indicating that HN2 inhibits the formation of liver tumors by azo dyes by blocking mitosis and the synthesis of desoxyribonucleic acid.

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Effect of Regeneration and Inanition on the Electrophoretic Properties of Soluble Rat Liver Proteins*

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The soluble proteins of 4-dimethylaminoazobenzene(DAB)-induced hepatoma, its mesenteric metastases, Jensen sarcoma, Flexner-Jobling carcinoma, and Walker 256 carcinosarcoma closely resemble one another electrophoretically (15). In comparison with preparations from (*a*) livers of rats fed stock or basal diets, (*b*) preneoplastic livers of rats fed DAB, and (*c*) non-neoplastic liver adjacent to DAB-induced hepatoma, the soluble proteins of the above tumors exhibit both a marked reduction in the amount of the slowly moving h components and a large increase in the fast moving components. The slowly growing transplantable mouse hepatoma 112B, however, was found to have h components intermediate in size between those of the above tumors and mouse liver. A subsequent study (18) revealed that the bulk of the protein-bound aminoazo dye(s) found in the livers of rats fed the 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), 4′-methyl-4-dimethylaminoazobenzene (4′-Me-DAB), and 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB) migrated electrophoretically with the proteins of the h components. These findings are compatible with an essential protein depletion mechanism of aminoazo dye carcinogenesis as suggested by Miller and Miller (11, 12).

To distinguish between the rate of growth, per se, and neoplasia as the cause of the electrophoretic differences described above, the present study concerned itself with the electrophoretic properties of the soluble proteins of non-neoplastic rat liver, in a state of rapid growth during regeneration (1, 4), on the one hand, and in a state of minimum growth during total inanition on the other (5, 6, 13).

Associated with studies on the metabolism of the liver proteins is the still unsettled question of the possible existence of a specific reserve or preferentially labile protein pool. Investigations which bear on this problem include those reviewed by Luck (8, 9), and Kosterlitz and Campbell (5), and those reported by Dumazert and Grac (2, 3). In general, these studies have dealt with the extent and nature of the loss of liver proteins in fasted or protein-deficient animals and the gain of liver proteins following the return to an adequate diet. Possible preferential liver protein depletion caused by the demands of rapid growth following partial hepatectomy and possible repletion of these liver proteins during the return to normal growth presents another experimental approach to the question of a reserve protein pool.

Therefore, in addition to the interest arising from the previous studies on neoplasia, it seemed desirable to apply the mild preparative and electrophoretic technic for the study of soluble rat liver proteins (16, 17) to an investigation of the possible existence of a preferentially labile liver protein pool. The results of the present study indicate that, both throughout a 9-day fast and throughout an 8-day period of regeneration following partial hepatectomy, the soluble rat liver proteins present essentially the same electrophoretic pattern as those of normal rat liver.

METHODS

Regenerating liver.—Prior to and throughout the experimental period, male albino rats of the Sprague-Dawley strain were fed Purina Dog Chow and distilled water ad libitum and were housed individually in screen-bottomed cages. Rats weighing between 235 and 270 gm. were partially hepatectomized[1] by the method of Higgins and Anderson (4), whereby approximately 70 per cent of the liver mass was removed (1, 4). Twelve hours, 1, 2, 4, 6, and 8 days after the operations, the number of rats given in Table 1 were sacrificed, and single pools of their soluble liver proteins were prepared for electrophoretic analysis.

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Inanition.—Male albino rats of the Sprague-Dawley strain, weighing between 250 and 280 gm. at the start of inanition, were previously fed Purina Dog Chow and distilled water ad libitum for at least 4 days and were housed individually in wide screen-bottomed cages. After fasts of 1, 2, 4, 7, and 9 days, throughout which times distilled water was available, the number of rats indicated in Table 2 were sacrificed and single pools of their soluble liver proteins were examined by electrophoresis.

Preparation and analysis of soluble rat liver proteins.—The soluble proteins of perfused rat livers were prepared and electrophoretically analyzed in the manner previously described (16, 17). Briefly, the procedure consisted of the perfusion of the livers with homogenization buffer, homogenization with a lucite pestle-type homogenizer in a sodium phosphate buffer at pH 7.8, 0.1 M phosphate, centrifugation at 15,000 × *g* for 15 minutes followed by 15,000 × *g* for 2–4 hours, and two periods of anaerobic dialysis of the clear supernatant fluids against sodium veronal buffer at pH 8.6, 0.1 μ, for a total of 15–24 hours. Electrophoresis in an 11-ml. cell for 180 minutes at 6.0–6.1 v/cm followed shortly thereafter. The protein concentrations (N × 6.25) of these supernatants were 2.2–3.3 per cent in the case of the fasted, and 3.1–3.6 per cent in the case of the hepatectomized animals. Photographs were taken with the Longsworth scanning optical system (7) and a tungsten filament lamp. Relative areas of symmetrically drawn curves (14) and mobilities from the starting boundaries are presented for the descending components, which are designated in a manner similar to those of normal rat liver (17).

Nitrogen analyses were performed by the micro-Kjeldahl method with selenium catalyst. Protein nitrogen was determined by the difference between the total nitrogen and the nonprotein nitrogen, which is soluble and nonsedimentable after 1 volume of sample has been mixed with 5 volumes of 10 per cent trichloroacetic acid.

RESULTS

The average weight loss of the rats after the several periods of inanition are given in Table 2. The amount and character of the soluble nitrogen fractions extracted from the homogenates of the regenerating livers and livers of fasted rats are indicated, respectively, in Tables 1 and 2.

The results of the electrophoretic analyses of the soluble proteins of regenerating livers and livers depleted by fasting are presented in Tables 1 and 2, respectively. For purposes of comparison, the average values and standard deviations of corresponding data for normal rat livers (17) are given in Table 1. Statistical analysis indicates that there is little significant difference between the electrophoretic data for the soluble liver proteins of the experimental animals and those of normal rats. However, mention should be made of the slight deviations of the a_2 and N components of regenerating liver and livers from fasted animals, respectively, from the corresponding components of normal rat liver.

DISCUSSION

The findings of the present study show that the h components are not affected by the rate of growth of non-neoplastic rat liver. The reduced h components observed in the soluble proteins of 4-dimethylaminoazobenzene-induced hepatoma and other tumors (15) may therefore be considered to be characteristic of neoplasia rather than of rapid growth per se. Similarly, the previously encountered marked increases in the sizes of the A and N components of tumors have not been observed in the present studies and therefore appear to be associated with neoplasia rather than with rapid growth.

The electrophoretic analyses of the soluble proteins of rat liver throughout regeneration and inanition indicate the absence among the soluble liver proteins of a specific or preferential reserve protein pool. Both regeneration and total inanition, with the resultant greater demands for liver proteins, would be expected to deplete the livers of possible specific reserve or preferentially labile soluble protein stores, with a consequent alteration in the relative amounts of the electrophoretic components. Since no significant change in the relative amounts of electrophoretic components was observed, it would appear that at least no one of the nine electrophoretic components observed is metabolically more labile than the others. These findings are in agreement with those of Luck (8, 10) and Dumazert and Grac (2, 3), who found that the proteins of various liver fractions were lost equiproportionately during fasting.

SUMMARY

Throughout an 8-day period of liver regeneration following 70 per cent partial hepatectomy and throughout a 9-day fast, electrophoretic analyses of the soluble rat liver proteins showed no significant difference from those of normal rat liver extracts.

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TABLE 1

ELECTROPHORETIC AND NITROGEN DISTRIBUTION DATA FOR NORMAL AND REGENERATING RAT LIVER EXTRACTS

DAYS AFTER PARTIAL HEPATECTOMY	No. RATS POOLED	SUPERNATANT N / HOMOGENATE N (PER CENT)	SUPERNATANT NPN / HOMOGENATE N (PER CENT)	AREAS, PER CENT Components i	h_2	h_1	g	b	a_2	a_1	A	N	MOBILITY $\times 10^{-5}$ CM^2/V SEC Components i	h_2	h_1	g	b	a_2	a_1	A	N
½	4	53.6	6.2	2.7	5.8	7.4	14.2	13.8	14.4	22.6	9.9	9.2	+0.80	−0.43	−1.09	−2.11	−3.17	−3.98	−4.91	−5.80	−6.85
1	3	54.2	6.7	2.5	6.6	8.6	15.1	14.8	14.1	18.6	10.7	9.1	+1.05	−0.30	−0.94	−1.94	−3.00	−3.82	−4.64	−5.61	−6.81
2	3	56.3	5.9	2.3	6.1	9.1	11.5	11.8	19.0	23.5	7.1	9.6	+0.99	−0.22	−0.88	−1.99	−2.96	−3.90	−4.84	−5.94	−6.99
4	3	52.5	5.4	1.9	6.6	9.0	13.0	11.7	19.8	23.8	11.5	2.9	+0.96	−0.42	−1.11	−2.13	−3.04	−3.92	−4.96	−6.11	−7.41
6	3	51.8	5.4	2.9	6.6	9.6	13.1	11.1	19.3	20.0	13.5	3.8	+0.76	−0.42	−1.08	−2.04	−2.98	−3.82	−4.85	−6.02	−7.22
8	2	50.8	4.9	2.8	4.6	11.6	12.5	12.1	20.2	21.4	12.6	2.8	+1.03	−0.18	−0.79	−1.88	−2.86	−3.67	−4.71	−5.95	−7.08
Normal rat liver	21	55.3	5.2	3.1	6.7	8.0	14.4	15.7	18.5	18.3	10.1	5.4	+0.83	−0.35	−0.99	−2.04	−3.01	−3.86	−4.71	−5.71	−6.87
S.D.*	n=7	(3.1)†	(0.3)†	±0.6	±1.0	±1.6	±4.5	±2.2	±3.0	±2.9	±1.7	±2.4	±0.15	±0.10	±0.09	±0.09	±0.13	±0.11	±0.16	±0.25	±0.27

* S.D. (standard deviation) $= \sqrt{\frac{\Sigma(d^2)}{n-1}}$.

† Average deviation $\left(\frac{\Sigma d}{n}\right)$ values from ref. 17.

TABLE 2

ELECTROPHORETIC AND NITROGEN DISTRIBUTION DATA FOR LIVER EXTRACTS FROM FASTED RATS

DAYS FASTED	No. RATS POOLED	Av. LOSS OF WEIGHT (GM.)	SUPERNATANT N / HOMOGENATE N (PER CENT)	SUPERNATANT NPN / HOMOGENATE N (PER CENT)	AREAS, PER CENT Components i	h_2	h_1	g	b	a_2	a_1	A	N	MOBILITY $\times 10^{-5}$ CM^2/V SEC Components i	h_2	h_1	g	b	a_2	a_1	A	N
1	2	26	42.7	2.9	1.3	10.0*	8.6	9.5	12.5	25.9	16.2	9.8	6.2	+1.11	−0.24	−1.17	−2.12	−2.98	−4.03	−5.00	−5.95	−6.99
2	2	42	49.6	3.6	2.0	5.4	8.0	9.6	14.3	22.8	21.6	9.6	6.7	+1.22	−0.09	−0.82	−1.79	−2.74	−3.64	−4.75	−5.76	−6.84
4	2	71	49.7	3.4	2.3	6.6	7.4	11.6	13.2	27.6	18.0	10.3	3.0	+1.05	−0.18	−0.87	−1.92	−2.82	−3.72	−4.73	−5.70	−6.66
7	3	79	52.3	3.8	2.2	5.6	7.8	12.4	12.6	22.4	20.6	12.7	3.6	+0.87	−0.31	−1.03	−2.10	−2.96	−3.82	−4.79	−5.96	−6.99
9	2	100	56.4	4.0	2.2	5.8	7.2	11.3	14.0	23.6	21.8	9.4	4.8	+0.94	−0.96	−1.71	−2.76	−3.56	−4.38	−5.34	−6.40	−7.27

* Affected by anomalous spiking of the descending h_2 component (see [17]).

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The Use of X-Ray and Nitrogen Mustard To Determine the Mitotic and Intermitotic Times in Normal and Malignant Rat Tissues*

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The relative importance of the times involved in the various phases of cellular proliferation is basic to problems of growth but has received little attention until recently. Data on the quantitative comparison of the rate of cellular proliferation of mammalian tissues have also been meager. In a previous report (16), an indirect method was presented for determining the time consumed in mitosis and in "resting" between mitoses by cells of mammalian tissues. The data from this experiment indicated that the time spent in mitosis was such an insignificant portion of the cell-doubling time that the rate of proliferation of normal cells has an inverse relationship to the length of the resting stage or interphase. If this relationship were found to apply also to neoplastic cells, chemotherapy of neoplasia might be rendered more effective by substances which would prolong the resting stage of neoplastic cells rather than only interfere with their mitosis. No extensive search has as yet been made for substances with such action, and few if any are known to exist.

The present study was undertaken (*a*) to extend the data of the previous report on normal mouse tissues to rat tissues, (*b*) to compare the time components of the mitotic process of neoplastic and normal cells, (*c*) to compare quantitatively the rate of cellular proliferation in various normal and neoplastic tissues, and (*d*) to determine whether or not the radiomimetic drug, methylbis(β-chloroethyl)amine hydrochloride, (HN2), a nitrogen mustard, has the same effect as x-ray on mitoses and whether it could be substituted for x-ray in this method of determination.

EXPERIMENTAL METHOD

The method for determining the length of time involved in mitosis depends upon the fact that ionizing radiation prevents cells from entering mitosis while allowing those already past mid-prophase to continue through their division in an apparently normal manner (4, 21, 24, 25). This blockade causes a progressive fall in the mitotic index (MI) (defined as the number of mitoses per cell and for convenience expressed as a whole number times 10^{-3}). The rate of fall in the mitotic index is dependent upon the time required for the cells already in mitosis to complete their division. Therefore, by determining the mitotic index of tissues at intervals following irradiation until a mitotic index of zero is reached, the mitotic time (MT) would be determined. In practice, however, a mitotic index of zero is rarely attained, because the initiation of mitosis by a few cells is not inhibited by the lower doses of radiation and because higher doses injure some dividing cells sufficiently to cause a pronounced slowing or complete arrest of mitosis. For this reason, it becomes necessary to extrapolate the slope of the decline of the mitotic index to zero. Since, with few exceptions, the mitotic index does not immediately begin to fall following radiation, it is also necessary to extrapolate the slope upward to intersect the line representing the normal mitotic index. This is done by drawing a line calculated by the sum of the least squares method to fit the experimental points. The time intercept between the point of intersection of this line with the normal value and with zero represents the mitotic time (Chart 1).

Since high doses of radiation damage some cells sufficiently to cause a slowing of division and low doses of radiation do not prevent some cells from entering mitosis, it is important to select a dose intermediate between these extremes, inasmuch as both extremes in dosage would tend to give results showing excessively long mitotic times. Several doses of radiation were therefore employed in this study. The dose giving the shortest mitotic time was considered to be the optimum for the particular tissue in question, and comparisons were made between tissues on the basis of time derived by employing this "optimum" dose.

From the mitotic index (MI) and the mitotic

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time (MT) expressed in minutes, the intermitotic or resting time (IT), in minutes, may be calculated from the formula IT = MT/MI (13). This holds only for stationary cell populations where the number of cells produced is balanced by the number removed or destroyed as in most normal adult tissues. In tissues showing exponential growth, e.g., neoplasms, the formula becomes IT = 0.693 MT/MI, the factor 0.693 being necessary because the number of cells in the tissues is increasing exponentially with time (12).

MATERIALS AND METHODS

Five hundred and fifty male Sprague-Dawley rats were used in these experiments. Before use, the animals were acclimatized to the conditions of the laboratory for 7–10 days. For the determinations on neoplasms, the Walker rat carcinoma 256

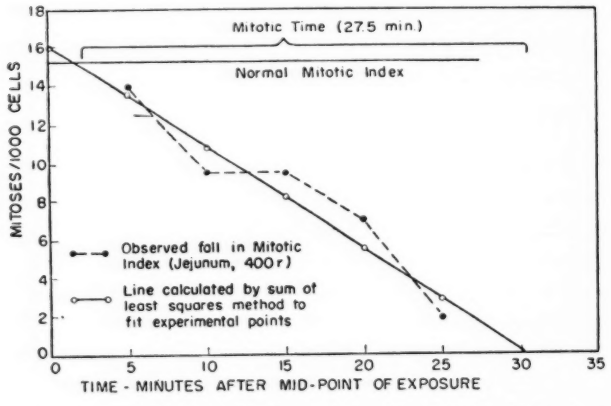
Mitotic Time (27.5 min.)
Normal Mitotic Index
MITOSES/1000 CELLS
Observed fall in Mitotic Index (Jejunum, 400 r)
Line calculated by sum of least squares method to fit experimental points
TIME - MINUTES AFTER MID-POINT OF EXPOSURE

CHART 1.—Method of determining length of mitotic time from changes in the mitotic index following x-irradiation.

and the Jensen rat sarcoma were used. Transplantation of the tumors was made subcutaneously in the manner previously described (11). Seven to 14 days after inoculation of the rats with one of the tumors, the animals were treated with either x-ray or nitrogen mustard. They were then killed at intervals following treatment, and the mitotic indices of the tissues were determined at each of the various time intervals.

Groups of rats were given doses of 200, 400, and 800 roentgens of x-ray in a single total-body exposure (250 kvP, 15 ma, inherent filtration equivalent to 2 mm. Al, 200 r/min). During exposures, the rats were confined in a flat lucite cage so constructed that shielding of one animal by the other was impossible. The radiation doses were measured in air with a 300 r Victoreen integron chamber placed in the center of the cage. Since tissues absorb ionizing radiations, determinations of the dose delivered to the site of the tumor implants and the jejunum were made by opening rats and placing the thimble of the ionization chamber adjacent to the tissue in question. A dose of 800 r to the skin had fallen to 797 r when measured at the site of the tumor and to 542 r at the site of the jejunum.

Rats receiving the nitrogen mustard were given doses of 0.8 or 4.0 mg/kg in physiological saline into the tail vein. Since it is known that nitrogen mustard reacts with the tissues in 5 minutes or less (15), the time required for this interaction to occur within the tissues is comparable to the time required to deliver the x-ray dosages.

The control animals were killed and autopsied just prior to sacrifice of the treated animals. All animals were sacrificed by crushing the skull, and the tissues were removed and fixed immediately. In the groups treated with x-ray, animals were killed at 5-minute intervals for 25 minutes from the mid-point of irradiation, and the following tissues were taken in this order: femoral marrow, Jensen or Walker tumor, and jejunum. The animals treated with 0.8 mg of nitrogen mustard/kg were killed at 5-minute intervals from the time of injection during the first hour and at 10-minute intervals during the second hour. The following tissues were removed in this group: Jensen or Walker tumor, and jejunum. In the group receiving 4 mg of nitrogen mustard/kg only sections of jejunum were taken. These were obtained by killing animals at 5-minute intervals for 50 minutes.

The bone marrow specimens were prepared from the femur by the method previously described (16). The remaining tissues were fixed either in Carnoy's fluid or in 10 per cent formalin, and paraffin sections 6 micra thick were prepared. With the exception of the bone marrow, which was stained with Wright's stain, all tissues were stained with Mayer's hematoxylin and counterstained with eosin or Fast green FCF.

The mitotic counts were done under an oil immersion lens. For the purposes of standardizing the counting technic, cells were arbitrarily considered to be in mitosis only between the appearance of recognizable elongation of the chromosomes in prophase and the final separation into two daughter cells. In the bone marrow, 1,000 cells per smear in both the myelocytic and erythrocytic series were counted, and the number of mitoses in each was determined. The number of mitoses per 100 suitable adjacent crypts of Lieberkühn were counted in the jejunum by the method of Dustin (7), after first determining the average number of cells per crypt by counting the total number of cells in 2,000 crypts. In the tumors, the incidence of mitoses per 650 cells was determined by the Chalkley method (6).

The mean value and standard error were calculated for the values obtained for each experimental point. Mitotic counts differing from the mean value by more than two standard deviations were excluded from the results. A minimum of eight animals per point was used for the x-ray studies. The minimum number of animals was reduced to 5 per point for the nitrogen mustard study without reducing the reproducibility of the results. Because the average value for the normal control animals was to be used in calculating the mitotic and intermitotic times, in addition to the mitotic index, it was desirable to have the value as statistically significant as practicable. Therefore, an average of sixteen animals was used to determine the control value.

RESULTS

The mitotic index of each tissue showed a fairly uniform decrease after x-radiation or nitrogen mustard injection. With the exception of the jejunum from the animals treated with nitrogen mustard, this decrease became manifest 5–10 minutes following treatment. The jejunum from animals treated with nitrogen mustard showed no rapid initial decrease as did the other tissues, but there was instead a somewhat slower fall beginning at 40 minutes. The changes in mitotic indices are shown in Charts 2–7. It can be seen from these graphs that in the animals treated with nitrogen mustard there was a sharp initial drop in mitosis in the tumors followed by a secondary increase in the count and then a second slow fall extending to

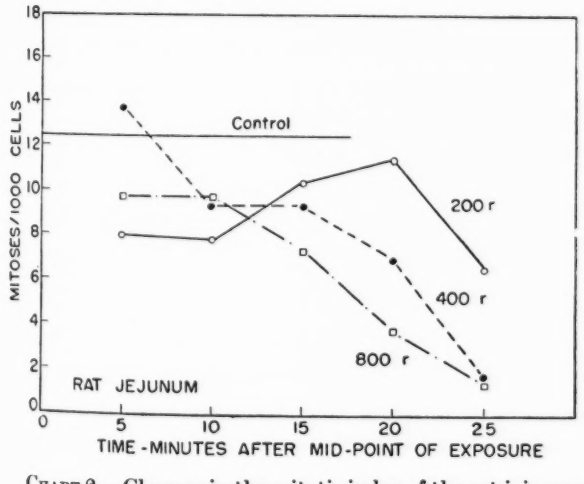

CHART 2.—Changes in the mitotic index of the rat jejunum at intervals following various doses of x-radiation.

the end of the observation period. The determinations on x-ray-treated animals were not carried on for long enough time intervals to find out whether a similar recovery and secondary fall were present.

In the initial decline of the mitotic index following treatment, the values for tissues of animals treated with nitrogen mustard did not reach as low levels as did the tissues of animals exposed to x-rays. This is probably due to an increase in the number of dividing cells damaged sufficiently to

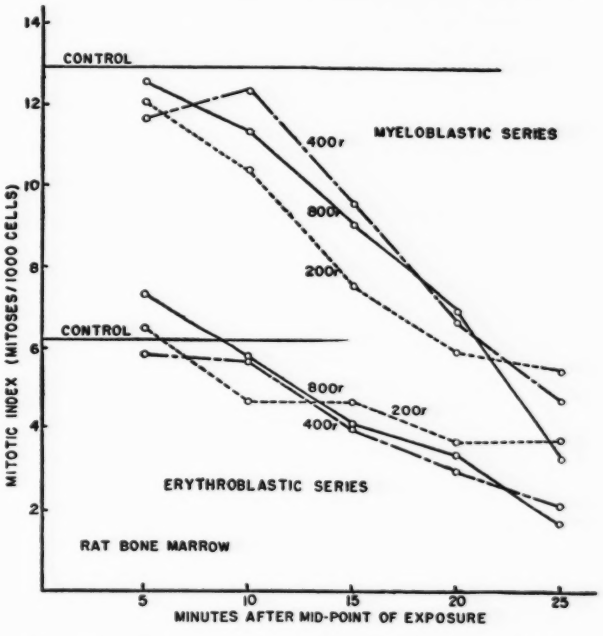

CHART 3.—Changes in the mitotic index of the myeloblastic and erythroblastic series in rat bone marrow following various doses of x-radiation.

slow or stop mitosis. More abnormal mitotic figures were seen in tissues from animals treated with nitrogen mustard than from animals treated with x-ray. These abnormalities consisted principally of prophases showing "stickiness" of chromosomes, metaphases lacking spindle fibers, and "bridging" of chromosomes in the late phases. Mitotic times were calculated on the basis of the initial rapid decline in the mitotic index using the method as described above (Chart 1). The times derived by using the various dosage levels are shown in Table 1. The shortest mitotic time obtained for each tissue is indicated by an asterisk in Table 1 and is listed separately in Table 2. These are considered to be the most accurate determinations for reasons discussed above. These times vary only from 25 to 27 minutes; therefore, the actual time spent in mitosis is probably fairly constant for a wide variety of rat tissues including experimental tumors. Furthermore, this time is so brief that it is of negligible importance in the cell-doubling time.

The intermitotic or resting times of cells of the various tissues were calculated from the formula $IT = MT/MI$ for the normal tissues and by the

formula $IT = 0.693\ MT/MI$ for the neoplasms. These times are listed in Table 1. In Table 2 the tissues are listed in the order of their relative rates of proliferation as indicated by the intermitotic

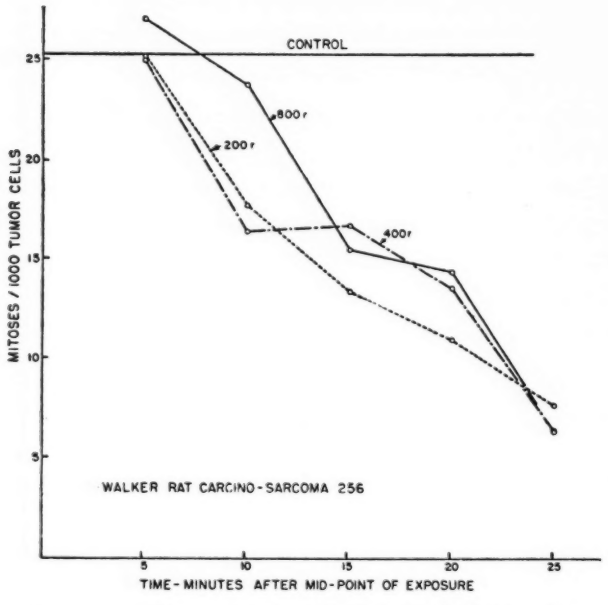

CHART 4.—Changes in the mitotic index of Walker rat carcinoma 256 following various doses of x-radiation.

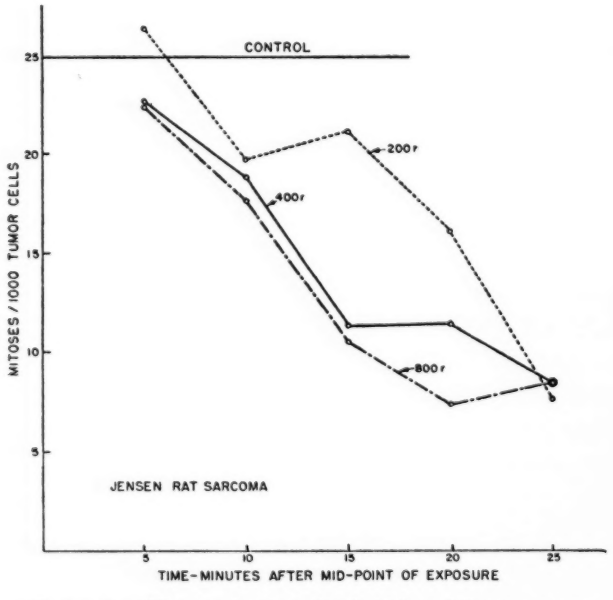

CHART 5.—Changes in the mitotic index of Jensen rat sarcoma following various doses of x-radiation.

times. As might be expected, the two tumors show the shortest resting time followed by the myelocytic series of the bone marrow and the jejunum, which are both approximately equal in their rates of proliferation, and finally by the erythrocytic series of the bone marrow.

DISCUSSION

The use of x-radiation to inhibit mitosis in early prophase appears to be a relatively simple *in vivo* method for the determination of how long a time the cells of various tissues spend in cell division. From the data obtained by this method,

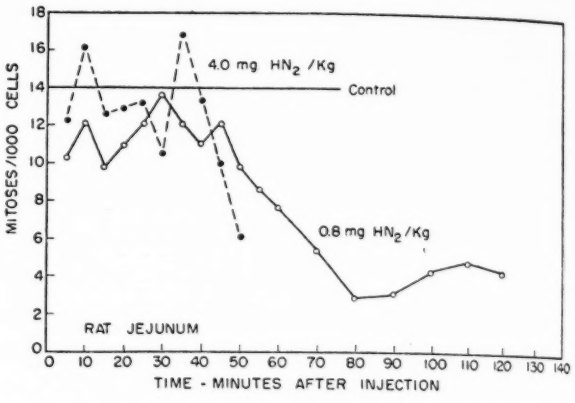

CHART 6.—Changes in the mitotic index of rat jejunum following treatment with nitrogen mustard.

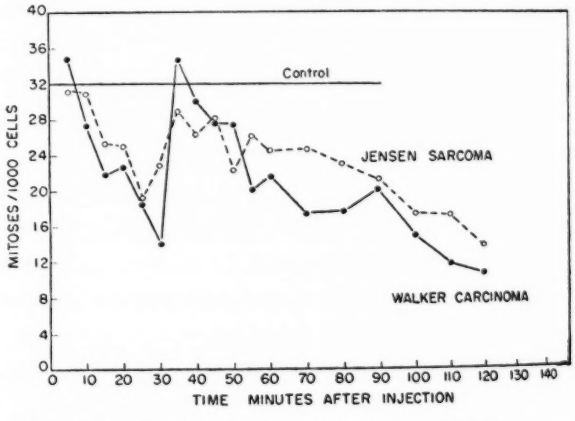

CHART 7.—Changes in the mitotic index of Walker rat carcinoma 256 and Jensen Sarcoma following treatment with nitrogen mustard.

all tissues of the rat that were examined seem to take essentially the same length of time to complete cell division. If the early portion of prophase, which cannot be measured accurately, is ignored, mitosis of normal and malignant rat tissues required 25.4 ± 0.9 minutes for completion. With this figure established for the rat, it is possible to substitute it as a constant in the formula for the derivation of the length of the resting stage so that the formula now reads: intermitotic time (minutes) = 25.4/number of mitoses per one cell, or $IT = 25.4/MI$. Where a tissue is growing exponentially, the formula is: $IT = 0.693\ (25.4)/MI$ or $IT = 17.6/MI$. Thus, in the case of the regenerating rat liver, where mitotic arrest could not be produced by amounts of radiation that were

feasible,[1] it is possible to determine the length of the resting stage by knowing the mitotic index at any time. At 46 hours after partial hepatectomy, the mitotic index was 9.0 per thousand cells or 0.009 mitoses per cell. Using the equation for exponential growth and solving for IT using this mitotic index, the resting stage of the regenerating hepatic cell after 46 hours was 1,955 minutes or 32.6 hours in length. Similarly, the length of the resting stage for cells of other tissues or tumors can now be found for the rat by determining the mitotic index and solving the above formulae.

The term "rate of cellular proliferation" is used commonly in oncology and in other studies of normal and abnormal growth, but it is rarely defined. While it is true that the rate of cellular proliferation is proportional to the mitotic index, the mitotic index is actually a measure of the length of the resting stage of cellular life and does not reflect the speed of the mitotic process, which is apparently constant. Thus, the pathologist's rule of thumb that the higher the mitotic index, the greater the rate of cellular proliferation of the tissue is correct, but actually is another means of expressing the concept that the shorter the resting stage of cellular life, the greater the number of cells in mitosis at any time. The numerous possible applications and uses of this concept in problems of cellular growth are obvious. For example, using the mitotic indices of the regenerating rat hepatic

[1] N. P. Knowlton and W. R. Widner, unpublished data, 1949.

TABLE 1

MITOTIC INDEX, MITOTIC TIME, AND INTERMITOTIC TIME OF RAT TISSUES AS DETERMINED BY USING VARIOUS DOSES OF X-RAY OR NITROGEN MUSTARD

Tissue	Treatment	Mitotic index	Mitotic time (minutes)	Intermitotic time (hours)
Jejunum	200 r x-ray	$(13.7 \pm 1.1)\ 10^{-3}$	105.9	129.2
	400 r x-ray		27.5*	33.5*
	800 r x-ray		29.8	36.4
	0.8 mg HN2/kg		54.9	66.9
	4.0 mg HN2/kg		20.8	25.3
Myelocytic series of marrow	200 r x-ray	$(13.2 \pm 0.6)\ 10^{-3}$	31.4	39.5
	400 r x-ray		25.7*	32.4*
	800 r x-ray		29.0	36.6
Nucleated red blood cells	200 r x-ray	$(6.5 \pm 0.4)\ 10^{-3}$	42.5	105.4
	400 r x-ray		35.4	87.7
	800 r x-ray		24.6*	61.0*
Walker rat carcinoma	200 r x-ray	$(25.2 \pm 1.6)\ 10^{-3}$	30.3	14.2
	400 r x-ray		33.4	15.4
	800 r x-ray		24.8*	11.4*
	0.8 mg HN2/kg	$(31.9 \pm 2.0)\ 10^{-3}$	42.6	15.5
Jensen rat sarcoma	200 r x-ray	$(25.0 \pm 2.3)\ (10^{-3}$	30.5	14.2
	400 r x-ray		28.4	13.2
	800 r x-ray		26.6*	12.3*
	0.8 mg HN2/kg	$(32.0 \pm 1.9)\ 10^{-3}$	62.7	22.7

* Indicates times considered most accurate for reasons discussed in the text.

TABLE 2

COMPARISON OF THE RATE OF PROLIFERATION OF MOUSE (16) AND RAT TISSUES AS INDICATED BY THEIR MITOTIC INDICES AND INTERMITOTIC TIMES

	Mouse			Rat		
Tissue	Mitotic index ($\times 10^3$)	Mitotic time (minutes)	Intermitotic time (hours)	Mitotic index ($\times 10^3$)	Mitotic time (minutes)	Intermitotic time (hours)
Walker carcinoma				25.2	24.8	11
Jensen sarcoma				25.0	26.6	12
Myelocytic series	3.8	35.3	155	13.2	25.7	32
Jejunum	9.3	23.9	43	13.7	27.5	33
Erythrocytic series	5.0	29.5	99	6.5	24.6	61
Ovary	2.8	21.1	123			
Lymph node	0.67	23.2	580*			
Epidermis	0.75	30.2	670 - 29 da			
Adrenal	0.22	14.4	1090			

* Recent experiments indicate this value should be about 100 hours.

cells, as published by Brues and Marble (2), and solving the formula above, it is seen that the resting time of the hepatic cells varies with the time after hepatectomy. At the end of 24 hours when the mitotic index was 0.0213 per cell, the intermitotic time was 13.8 hours; at 48 hours: mitotic index, 0.0097, and intermitotic time, 30.2 hours; and at 72 hours: mitotic index, 0.0063, and intermitotic time, 46.6 hours. In the normal liver, where one mitosis is seen among 20,000 cells, the resting stage is by these calculations 8,466 hours or 353 days in length. Stimulants to increased rate of cellular proliferation appear to affect this change by shortening the intermitotic time. Thus, the rat bone marrow when stimulated by subcutaneous injections of turpentine undergoes myelocytic hyperplasia, the mitotic index is increased, the time involved in visible mitosis remains 25.7 minutes, and the resting stage of the myelocytes is decreased from 32.4 to 24.9 hours.[2]

The application of the concept of the importance of the resting stage in growth to problems in oncology and chemotherapy of malignant tumors may be fruitful. For example, in a study of the effect of urethan on Walker rat carcinoma 256 (11), Green and Lushbaugh concluded that urethan inhibits cellular proliferation in part by nutritional debilitation of the animal and by increasing the production of nonproliferating daughter cells. Restudying these data with the concepts and formulae described here, it is found that the resting stage of the carcinoma cells was 14.6 hours in normal well fed animals but 32.6 hours when growing in animals that had been depleted of their proteins for 3 months. In urethan-treated rats, force-fed in an attempt to overcome the starvation that accompanies this drug, the resting stage was 20.9 hours in length. Thus, it would seem from these observations of changes in the length of the resting stage that a possible mode of action of urethan on this tumor was to prolong the time between mitoses. Further study of the length of the resting stage of other types of tumors in the rat and other animals and its modification by drugs would seem to be a potentially profitable avenue of approach to the chemotherapy of cancer.

The accuracy of this method for determining the mitotic time must be considered. While it is possible that the rate of completion of mitosis by cells exposed to x-radiation is slowed, giving unduly long values for the mitotic time, the evidence of other investigators indicates that this is not so. There may be a pronounced delay in the completion of prophase at the time of irradiation, but most cells past the stage of breakdown in the nuclear membrane complete division at a normal rate over wide ranges of radiation dosages (4, 21, 24, 25). The slight delay in the fall of the mitotic index is most likely due to the fact that inhibition of mitoses occurs at a stage slightly earlier than the arbitrary limit set as the beginning of prophase for the purpose of counting. The mitotic times measured by this method did not represent the total time required for a cell to divide, but only that time required for cells to complete division from the stage of recognizable prophase. The time required for the cells to go through early prophase to the point of recognizable elongation of the chromosomes, therefore, could not be taken into account. This time has been estimated variously to take 30 minutes to 2 hours (3, 5, 14, 18, 19). Since the mitotic index also does not include cells in this early part of prophase, the intermitotic time derived from the division of the mitotic time by the mitotic index should be an accurate value, because the error cancels out in the formula $IT = MT/MI$.

The mitotic times obtained in this experiment compare closely with those obtained by other investigators using different methods of determination. A comparison of these times with those reported by other investigators is made in Table 3. The majority of the times derived from tissue cultures were made by direct observation of mitosis. Part of the variation in the times is due to differences in the definition of the limits of mitosis. Variable portions of prophase are included, and there is poor agreement as to when telophase ends. Only in the *in vivo* experiments with mice and rats in which x-ray was used as the mitotic inhibitor is the same portion of mitosis being considered. Despite these variations, it is obvious that the mitotic times are of similar magnitude for a wide variety of tissues as determined by many different technics.

A comparison of the mitotic and intermitotic times of the various tissues studied in this and a previous report (16) is given in Table 2. It is believed that the mitotic and intermitotic times for the rat tissues are more accurate than those given for the mouse tissues, because animals were killed at 5-minute rather than 10-minute intervals; thus, more points from which to calculate the times were available. In addition, three doses of x-ray were used rather than one, and the "optimum" dose was used in calculation of the mitotic and intermitotic times for the rat tissues. The value for the mouse lymph node might be in error. More recent experiments on mice have indicated

[2] C. C. Lushbaugh, unpublished data, 1950.

that the intermitotic time of lymphocytes is actually closer to 100 hours.[3]

Nitrogen mustard is not a satisfactory substitute for x-ray in this method of determining mitotic times. In every case except that of the jejunum from the rat receiving 4.0 mg/kg, the mitotic times derived were longer than those derived by using x-ray. The differences in fall of the mitotic index were probably caused by a difference in the mode of action on mitosis of the two agents. It has been suggested by other investigators that nitrogen mustard exerts its inhibitory effect on the mitotic cycle in the premitotic stage (1, 8, 10, 20, 22), which would delay the fall of the mitotic count following treatment. Such a delay was found in the jejunum and tumors of the rats treated with nitrogen mustard. The tumors also showed an early rapid fall similar to that produced by x-ray as well as the delayed fall. This is believed to indicate a second point of mitotic arrest by the nitrogen mustard. Despite the similarity of the decline of the mitotic index for the tumors treated with nitrogen mustard and the tissues treated with x-ray, the times derived by the use of the nitrogen mustard were longer than those derived by using x-ray because of the increased number of damaged cells in which mitosis was stopped. This "colchicine-like" effect has been described by Friedenwald *et al.* (9). Because of this increased mitotic time, the intermitotic times for the tumors of animals treated with nitrogen mustard were longer than for those of animals treated with x-ray.

[3] J. B. Storer, unpublished data, 1951.

SUMMARY AND CONCLUSIONS

1. By determining the rate of decline of the mitotic indices of the jejunum, bone marrow, Jensen sarcoma, and Walker rat carcinoma 256 of rats immediately following exposure to x-radiation, it was found possible to determine the length of time spent by the cells in mitosis and in the resting stage. Comparison of the results of this experiment with those of others revealed a close agreement between the mitotic times for a wide variety of tissues, derived by several different methods.

2. From these data it would appear that the

TABLE 3

COMPARISON OF THE TIME REQUIRED FOR MITOSIS BY CELLS OF VARIOUS TISSUES

Animal	Tissue	Method	Mitotic time (minutes)	Author
Chick	"Embryonic"	Tissue culture	18–20 (av.)	Levi (18)
	Fibroblasts	" "	20 (av.)	Simon-Reiss and Spear (23)
	"	" "	15–40	Juul and Kemp (14)
	"	" "	25	Willmer (26)
	Heart	" "	34 (av.)	Wright (27)
	Mesenchyme	" "	32 (av.)	Lewis and Lewis (19)
Cat	Connective Tissue	" "	15–30	Lambert and Hanes (1913) cited by Lewis and Lewis (19)
Rat	" "	" "	25–45	*Ibid.*
	" "	" " (38°–39° C.)	21–29	Lambert (1913) cited by Lewis and Lewis (19)
	" "	" " (34°–36° C.)	35–50	*Ibid.*
	Cornea	*In vivo* using ether to inhibit mitosis	70	Buschke, Friedenwald, and Fleischmann (3)
	"	Same as above	34.3*	*Ibid.*
	Jensen rat sarcoma	*In vivo* using radium to inhibit mitosis	38*	Mottram, Scott, and Russ (21)
	Walker rat carcinoma	*In vivo* using x-ray to inhibit mitosis	24.8	Table 2, this paper
	Jensen rat sarcoma	Same as above	26.6	" " " "
	Marrow, myeloid series	" " "	25.7	" " " "
	Marrow, erythroid series	" " "	24.6	" " " "
	Jejunum	" " "	27.5	" " " "
Mouse	Jejunum	" " "	23.9†	Knowlton and Widner (16)
	Marrow, erythroid series	" " "	29.5†	" " "
	Marrow, myeloid series	" " "	35.3†	" " "
	Ovary	" " "	21.1†	" " "
	Lymph node	" " "	23.2†	" " "
	Epidermis	" " "	30.2†	" " "
	Adrenal	" " "	14.4†	" " "

Note: Times derived from tissue culture were from direct observation or by repeated photographs.

* Times calculated by us from other authors' data by the method described in this paper.

† Times derived by Knowlton and Widner (16) by modification of the method described in this paper.

time required for any cell of the rat to produce two daughter cells following breakdown of the nuclear membrane in early prophase is constant and measures approximately 25.4 minutes. It would also appear that the mitotic index of a tissue is dependent upon the length of the interphase or resting stage and that changes in rate of cellular proliferation are the result of changes in the length of the resting stage.

3. It was not found possible to substitute nitrogen mustard for x-radiation as a means of producing mitotic arrest in this method of determining mitotic time, because nitrogen mustard apparently causes an early premitotic arrest as well as an arrest in early prophase, while also having a colchicine-like effect later in mitosis. These differences in mode of action of nitrogen mustard upon cell division lead to unduly prolonged mitotic times as derived by this method.

ACKNOWLEDGMENTS

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The Distribution of Radioactivity in Mice Following Administration of 3,4-Benzpyrene-5-C^{14} and 1,2,5,6-Dibenzanthracene-9,10-C^{14}*†

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The extensive investigations into the distribution and excretion of benzpyrene by fluorescence measurements have served to establish a number of facts, which, however, are only qualitative in nature. In particular, the investigations of Weigert, Mottram, and Calcutt (2, 11–13) have cast considerable light upon the metabolic transformations of the hydrocarbon in the skin. It has previously been shown (5) that a single dose of labeled dibenzanthracene-9,10-C^{14} may be quantitatively accounted for by measurements of the distribution and excretion of radioactivity, and considerable information may be gained as to the chemical nature of the metabolites by systematic chemical fractionations (6). Accordingly, in order to obtain a basis for quantitative comparisons with dibenzanthracene, a study of the distribution of radioactivity following intravenous and subcutaneous injection of benzpyrene-5-C^{14} into mice has been carried out (7). A direct comparison has also been made of the rates of disappearance of benzpyrene-5-C^{14} and dibenzanthracene-9,10-C^{14} from the site of a single application to the skin.

EXPERIMENTAL

All C^{14} determinations were carried out in internal flow counters and are corrected for self-absorption. All samples were counted for a sufficiently long time to achieve at least 10 per cent accuracy. Homogenates, feces, and tissue residues were combusted in duplicate by standard procedures, using the Van Slyke and Folch reagent. Tissue extracts were plated in duplicate by evaporation onto aluminum discs (3). All feces were dried in a vacuum oven, pulverized, and passed through a 50-mesh screen.

All the mice were females of the Rockland strain. For intravenous injection, the benzpyrene was converted into an aqueous colloid, as described for dibenzanthracene, and injected into the tail vein (5). The animals were maintained in all-glass metabolism cages. The respiratory CO_2, urine, and feces were collected, and at the end of the appropriate time the animals were sacrificed, dissected, and the tissues individually pulverized in liquid air and dried in the vacuum oven at 60°. The residues were combusted for radioactivity determinations. It was shown in a separate experiment that the radioactivity in the "carcass + bile" category was present almost exclusively in the gall-bladder and bile.

For subcutaneous administration, the benzpyrene was dissolved in tricaprylin (0.5 mg/0.2 ml) and injected into the sacral region. In spite of all precautions, some leakage occurred. The feces were collected during the experiment, and at appropriate times the animals were sacrificed, and the fluorescent site of skin and surrounding muscle was dissected and fractionated as described below.

To study the effect of applying the carcinogen to the skin, the skin from the sacral region was shaved, and a solution of benzpyrene-5-C^{14} in benzene (152,000 c/m, 0.05 mg) in one series and of dibenzanthracene-9,10-C^{14} (28,700 c/m, 0.05 mg) in benzene in another was applied with a micropipet. The mice were confined in the cages illustrated in Figure 1, and the feces were collected. The cages were constructed from coarse wire screening, and mice could be maintained in them with adequate food and water for up to 21 days. The "chimneys" used to protect the sites of application from being rubbed against the cages were prepared by cutting 5-mm. sections of 10×30 mm. plastic centrifuge tubes. A small flange was obtained by heat treatment, and the collar was fastened to the mouse with adhesive tape and collodion. In this arrangement, the mice could not lick the sites where the carcinogen had been ap-

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plied. These sites were dissected and fractionated in the same manner as the subcutaneous areas.

The fractionation procedure is shown in Chart 1. The tissue was pulverized in liquid air in a stainless steel mortar and pestle and homogenized in 10 per cent trichloroacetic acid. The homogenate was transferred to a round-bottom flask fitted with a moisture trap and reflux condenser. The mixture was boiled with benzene until no more water was collected, and then 1 hour longer. The subsequent extractions were all carried out in centrifuge tubes and with the aid of capillary medicine droppers. The solutions were plated directly and counts were made.[1]

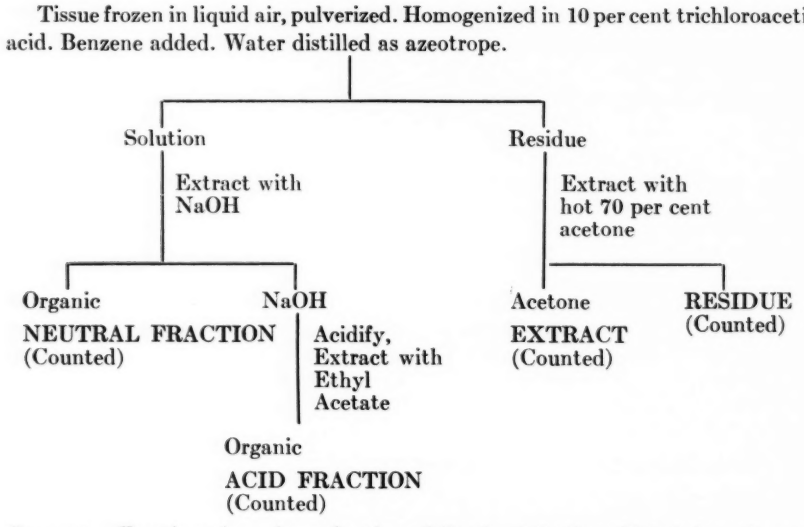

CHART 1.—Fractionation scheme for tissue following injection of labeled carcinogens

RESULTS

Intravenous injections.—The results of the experiments on the intravenous injection of an aqueous colloid are shown in Table 1, and it may readily be seen that the dose is accounted for quantitatively. There is no radioactivity in the respiratory CO_2, indicating that the carcinogen is metabolized in such a way that the labeled carbon atom 5 does not enter into many of the physiological metabolic pathways of the mouse.

The data resemble those obtained previously for dibenzanthracene (5) in several respects, although there are important quantitative differences. The pattern of excretion following this mode of administration is similar with both hydrocarbons; there is a rapid uptake by the liver, followed by an emptying through the bile into the gastrointestinal tract, and thence into the feces. Whereas, in the case of dibenzanthracene, the liver contained 89 per cent of the radioactivity at 90 minutes, in the benzpyrene-treated animal only 1.36 per cent of the radioactivity was in the liver and 72 per cent was in the bile at the same time. This more rapid metabolism of benzpyrene appears to occur after administration by all the routes that have been tested. Radioactivity from benzpyrene is also absorbed more rapidly into the body fats than that from dibenzanthracene. This increased metabolic rate may be due, to some extent, to the greater solubility of benzpyrene in water.

This excretion through the liver, bile, gastrointestinal tract, and feces following intravenous injection is not at all unique to carcinogenic hydrocarbons. The same pattern has been observed with various inorganic colloids, bromoestradiol, stilbestrol, and various steroid hormones. Thus, it would appear that this type of excretion, particularly in rats and mice, is largely determined by the physical and chemical, rather than the physiological, properties of the molecules.

Because of the similarity of the results with benzpyrene and dibenzanthracene, it was decided not to compare the two compounds following intraperitoneal or stomach-tube administration. Because of the evident lack of relationships between these routes and the process of carcinogenesis, it was decided to extend the investigation to subcutaneous injection and application to the skin, where some direct effects on carcinogenesis might be observed.

Rate of disappearance of radioactivity from the site of subcutaneous injection.—The rate curves of disappearance from the site of subcutaneous injection are given in Chart 2. The data on benzpyrene

[1] We wish to express our gratitude to Mr. Walter G. Wiest for the construction and design of the cages; and to Mrs. Edith Wallestad, Miss Nancy Lake, Mrs. Carolyn Kolterman, and Mr. Art Malmon for carrying out the radioactivity determinations.

were obtained in the present study. Individual mice are shown, and the values represent the sum of the radioactivities in the individual fractions described below. The accumulated C^{14} in the feces is also shown. The data on dibenzanthracene are taken from Heidelberger and Jones (5); the values on methylcholanthrene from Dauben and Mabee (4).

In these three studies, the dose and the solvents are identical, 0.5 mg. of carcinogen in 0.2 ml. of tricaprylin. However, in the experiments with dibenzanthracene and methylcholanthrene, A strain male mice were used, while in the benzpyrene study Rockland strain females were the experimental animals. In spite of this difference, we feel that a comparison of the three carcinogens is worth while.

TABLE 1

DISTRIBUTION OF RADIOACTIVITY FOLLOWING INTRAVENOUS INJECTION OF AN AQUEOUS COLLOID OF 0.45 MG. BENZPYRENE-5-C^{14}

	90 MIN.			24 HRS.		
	Dry wt. (gm.)	Total c/m*	Per cent of dose	Dry wt. (gm.)	Total c/m*	Per cent of dose
Dose		1,390,000			1,210,000	
Respiratory CO_2		<400	<.03		<100	<.008
Feces	None			0.575	794,000	65.5
Urine		3,300	0.24		210,000	17.3
Gastroinestinal tract + contents	0.834	163,000	11.7	0.532	16,600	1.37
Carcass fat + tissue	1.746	133,000	9.6	0.982	14,200	1.17
Abdominal fat + tissue	1.778	14,000	1.01	0.971	16,100	1.33
Liver	0.371	18,900	1.36	0.270	20,900	1.72
Kidneys	0.083	4,300	0.31	0.078	4,100	0.34
Lungs	0.043	1,000	0.072	0.039	890	0.071
Heart	0.029	480	0.034	0.031	350	0.029
Stomach	0.142	2,000	0.14	0.066	3,700	0.31
Salivary glands	0.029	300	0.022	0.035	540	0.045
Plasma	0.085	3,000	0.22	0.072	1,700	0.14
Red cells	0.178	310	0.022	0.179	590	0.049
Carcass + bile and gall bladder	7.038	1,000,000	72.0	5.614	92,100	7.60
Recovery		1,344,000	97.0		1,176,000	97.2

* Counts per minute.

With dibenzanthracene and benzpyrene the straight-line logarithmic plot, when extrapolated to zero time, comes out to well below 100 per cent. It is likely that this discrepancy is due to initial leakage from the sites of injection. Consequently, the half-time calculations were all based on the linear portion of the curve.

It is quite apparent from the data that the rate of disappearance increases in this sequence: dibenzanthracene, methylcholanthrene, and benzpyrene. It is also clear that the fecal excretion of radioactivity accounts almost completely for the loss of C^{14} from the site of injection in the benzpyrene experiment. Similar results have been obtained for the other two carcinogens (4, 5). At the dosage used in these experiments, methylcholanthrene produces a high incidence of tumors more rapidly than dibenzanthracene, and, on the basis of this fact, Dauben and Mabee (4) suggested that the rate of metabolism may play a role in tumor production. While this suggestion undoubtedly has some elements of truth, the results with benzpyrene do not support a direct correlation of carcinogenic effectiveness with rate of metabolism (as measured by the present criteria). Although benzpyrene is eliminated more rapidly than either dibenzanthracene or methylcholanthrene, its carcinogenicity under these conditions is less than that of methylcholanthrene yet greater than dibenzanthracene.

Perhaps a more valid interpretation of these results may be obtained from a consideration of the work of Bryan and Shimkin (1). These investigators carried out definitive experiments on a comparison of the carcinogenicity of these same three hydrocarbons when injected subcutaneously in tricaprylin into very large numbers of mice, and subjected their results to a rigorous statistical scrutiny. Without going into the details of their work, it may be stated that the following general conclusion pertinent to our experiments was reached: at minimal dosage of the carcinogens, the order of increasing carcinogenic potency was benzpyrene, methylcholanthrene, and dibenzanthracene. This is the inverse of the order of the rates of disappearance found in these studies.

If it is permissible to extrapolate the data of Bryan and Shimkin, obtained with C3H mice, to the present studies, an interpretation may be

made. Since at very low doses dibenzanthracene is the most carcinogenic, it is reasonable to conclude that this is due to its slow rate of elimination. Because benzpyrene is so rapidly eliminated, it is likely that under minimal dose conditions sufficient hydrocarbon is not present to initiate and promote the carcinogenic process, whereas there would be considerably more dibenzanthracene available for this purpose.

If this view is valid, it would appear that the interpretation of the data in the literature, such as those of Bryan and Shimkin (1), would have to be modified. Thus, the fact that dibenzanthracene was judged to be the most potent of the three hydrocarbons was the result of its longer period of retention at the active site. Indeed, since benzpyrene and methylcholanthrene are eliminated so much more rapidly, and yet produce a high incidence of tumors at short latent periods, it must be concluded that they are considerably more carcinogenic. Perhaps the interpretation of data obtained with relatively high doses may be considered valid, but any attempts to define the carcinogenic power of compounds at low dosages, without taking into account the rate of excretion, may result in errors of interpretation.

Rate of disappearance of radioactivity from the site of a single application to the skin.—The rates of disappearance of radioactivity from the site of a single application of benzpyrene-5-C^{14} and of dibenzanthracene-9,10-C^{14} are shown in Chart 3. It is evident that benzpyrene or its metabolites are eliminated much more rapidly than dibenzanthracene, and that the excretion is in the feces. This parallels the data obtained after subcutaneous injection.

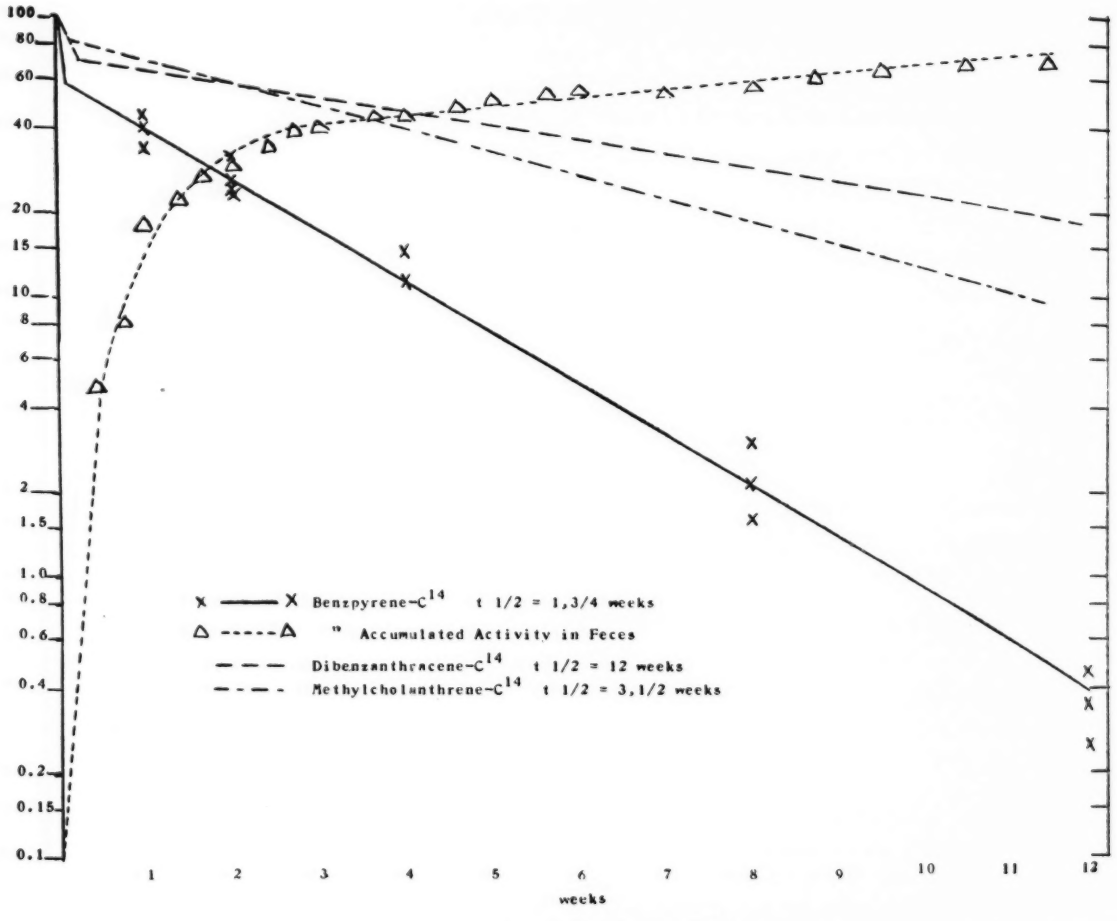

CHART 2.—Rate of disappearance of radioactivity following subcutaneous injection of carcinogenic hydrocarbons. The dibenzanthracene data is from Heidelberger and Jones (5). The methylcholanthrene data is from Dauben and Mabee (4).

It is believed that this and related work[2] represent the first unequivocal proof that hydrocarbons can be absorbed through the skin and eliminated.

In previous work, the skin of the mouse was always accessible, and it has been pointed out (2) that the mice lick and rub the carcinogen against the cage. As has previously been shown (5), orally

[2] Heidelberger and Wiest, unpublished experiments.

ingested dibenzanthracene is rapidly eliminated in the feces. In our experiments, this difficulty has been circumvented by the use of restriction cages, so that the rates of elimination we have obtained represent true rates, in the absence of extraneous factors. To our knowledge, such cages have not been previously used in studies of this type.

The elimination of the dibenzanthracene radioactivity is extremely slow, and no C^{14} could be detected in the feces. It has already been shown[2] that caloric restriction increases the rate of dibenzanthracene elimination. In this study,[2] synthetic diets were used, and it was possible to make radioactivity measurements because of the smaller bulk of feces. It appeared that about 1 per cent of the initial activity was excreted per day; this would represent a faster rate than that found in the present investigation, where a grain diet was used, and the large bulk of feces made accurate determinations of small quantities of C^{14} impossible.

In an interesting study, Simpson and Cramer (10) investigated the histological distribution in mouse skin of methylcholanthrene by fluorescence microscopy. They found that the carcinogen dissolved in the sebum and went to the sebaceous glands, which then degenerated. The fluorescent materials were then excreted into the hair follicles and into the lipids of the keratin layer. The sebaceous glands were destroyed in 4–5 days. It seems possible that the change of rate observed with benzpyrene may be caused by a similar phenomenon.

When dibenzanthracene was administered, considerable scaling of the epidermis was observed after 10 days. This scaly material was highly fluorescent, indicating that carcinogen was removed from living skin in this way, thus contributing to the slow rate of elimination.

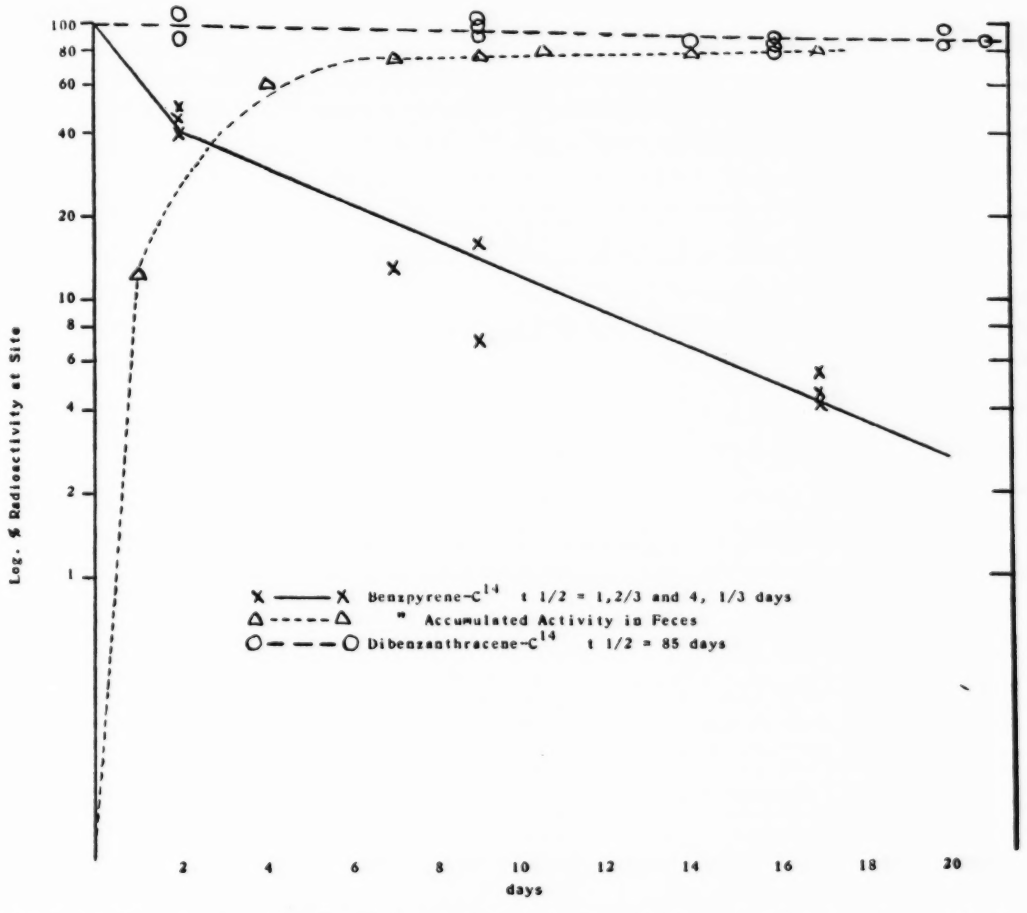

CHART 3.—Rate of disappearance of radioactivity from skin following a single application of carcinogenic hydrocarbon

Iball (8) has summarized the evidence showing that benzpyrene is a considerably more potent carcinogen than dibenzanthracene on application to the skin. Although insufficient data are at hand to draw conclusions concerning the correlation of carcinogenic activity and rate of elimination, it may be tentatively suggested that there is a direct relationship between these two factors in skin

painting, whereas there appears to be an inverse relationship in subcutaneous injections of small doses of carcinogen.

Fractionations.—The scheme for fractionation of the tissue sites following subcutaneous injection or application to the skin of the radioactive carcinogens is shown in Chart 1. The neutral fraction contains unchanged hydrocarbon and neutral metabolites, and the acid fraction contains carboxylic acids and phenols in the free state. The hot acetone extraction was carried out according to Weigert and Mottram (12) and should release material that is loosely bound to tissue constituents.

The discovery by Miller (9) that a portion of benzpyrene is bound to skin protein represents an important advance in research on the mechanism of hydrocarbon carcinogenesis. Although her fractionation procedure was considerably different from that of the present work, it appears very likely that radioactivity of the tissue residue, obtained by our method of fractionation, also represents carcinogen or a metabolite bound to protein.

The fractionation data following subcutaneous injection (Table 2) show that, in contrast to the results obtained with dibenzanthracene, benzpyrene is rather extensively metabolized under the skin. In particular, a considerable quantity of acidic or phenolic metabolites is produced in the first week; in fact, the disappearance of the acidic or phenolic radioactivity accounts almost entirely for the loss of total activity during the second week, since the C^{14} in the neutral fraction changes little in that time interval. The acetone extract also decreases progressively with time. The bound radioactivity in the residue appears to be relatively constant for the first 4 weeks, and then diminishes.

Results of applying the same fractionation procedure to the skin following a single application of labeled benzpyrene dissolved in benzene are shown in Table 3. There is a relatively small quantity of C^{14} in the acidic and phenolic fractions and acetone extracts, and, although there is a large C^{14} content in the neutral fraction, no information is yet available concerning its chemical nature. The percentage of the dose in the tissue residue is low, but sufficient radioactivity is present to make further experiments with this fraction entirely feasible.

Exactly comparable data obtained with labeled dibenzanthracene are presented in Table 4. Once

TABLE 2

COUNTS PER MINUTE IN FRACTIONS, BENZPYRENE-5-C^{14}, SUBCUTANEOUS

(The figures in parentheses are the per cent of the dose)

Mouse no.	Time (weeks)	Fraction			
		Neutral	Acid	Extract	Residue
1	1	605,000 (28.6)	164,000 (9.6)	11,500 (0.56)	11,100 (0.53)
2	1	306,000 (21.7)	162,000 (11.7)	7,120 (0.50)	10,800 (0.77)
3	1	410,000 (29.1)	204,000 (19.5)	10,900 (0.78)	15,300 (1.09)
4	2	383,000 (27.2)	34,400 (2.5)	3,200 (0.23)	5,830 (0.42)
5	2	338,000 (23.9)	4,500 (0.33)	2,200 (0.16)	3,730 (0.27)
6	2	317,000 (22.3)	2,800 (0.20)	2,700 (0.19)	10,600 (0.76)
7	4	256,000 (18.2)	50,700 (3.6)	3,600 (0.26)	30,000 (1.05)
9	4	127,000 (9.0)	71,700 (5.4)	850 (0.06)	3,500 (0.25)
10	8	21,600 (1.4)	5,500 (0.39)	160 (0.012)	590 (0.035)
11	8	37,600 (2.3)	10,400 (0.74)	100 (0.007)	1,050 (0.075)
12	8	12,100 (0.86)	430 (0.030)	160 (0.01)	2,800 (0.16)
13	12	4,600 (0.32)	1,200 (0.09)	120 (0.009)	590 (0.05)
15	12	1,490 (0.01)	530 (0.04)	40 (0.002)	1,100 (0.07)

TABLE 3

COUNTS PER MINUTE IN FRACTIONS, BENZPYRENE-5-C^{14}

SINGLE SKIN APPLICATION

(The figures in parentheses are the per cent of the dose)

Mouse no.	Time	Fraction			
		Neutral	Acid	Extract	Residue
7	4 hrs.	150,000 (98.6)	70 (0.05)	150 (0.10)	360 (0.24)
1	2 days	54,900 (36.0)	840 (0.55)	360 (0.24)	1,400 (0.92)
2	2 "	63,800 (41.9)	170 (0.11)	180 (0.12)	805 (0.53)
3	2 "	62,100 (40.7)	180 (0.12)	240 (0.16)	670 (0.44)
5	7 "	16,500 (10.8)	230 (0.15)	320 (0.21)	990 (0.65)
4	9 "	22,500 (14.8)	290 (0.19)	255 (0.17)	780 (0.51)
6	9 "	10,100 (6.7)	232 (0.15)	260 (0.17)	550 (0.36)
8	17 "	14,900 (5.0)	220 (0.07)		1,660 (0.56)
9	17 "	10,900 (3.6)	370 (0.12)		1,010 (0.34)
10	17 "	11,000 (3.6)	333 (0.11)		2,380 (0.79)

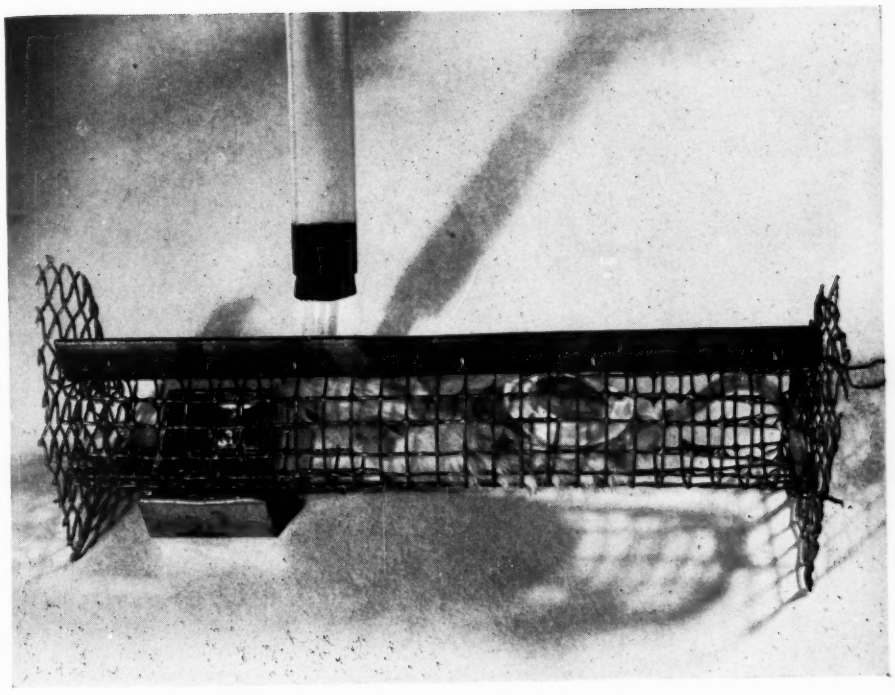

FIG. 1.—Restriction cage for mouse

again, it is evident that dibenzanthracene is strikingly less active metabolically than benzpyrene. The residue fraction contains a somewhat lower percentage of the dose than the similar fraction obtained from benzpyrene-treated mice.

These results of tissue fractionations following treatment with labeled carcinogenic hydrocarbons furnish quantitative information on the general nature of the metabolites produced at the site of carcinogenesis. They also confirm the observation of Miller (9) of protein-bound metabolites of benzpyrene in the skin and extend them by demonstrating a similar phenomenon in the case of dibenzanthracene.

It is hoped that these general observations on the rates of metabolism of carcinogenic hydrocarbons will lay the groundwork for further studies, now in progress, that will be concerned with more detailed aspects of the mechanism of hydrocarbon carcinogenesis.

TABLE 4

COUNTS PER MINUTE IN FRACTIONS, DIBENZANTHRACENE-9,10-C^{14}

SINGLE SKIN APPLICATION

(The figures in parentheses are the per cent of the dose)

Mouse no.	Time (days)	Fraction			
		Neutral	Acid	Extract	Residue
1	2	23,200 (81.0)	270 (0.94)	30 (0.10)	66 (0.23)
2	2	29,600 (103)	0 (0)	30 (0.10)	72 (0.25)
3	2	26,000 (90.5)	47 (0.16)	100 (0.35)	16 (0.05)
4	9	26,800 (93.5)	0 (0)	110 (0.38)	96 (0.34)
5	9	23,700 (82.5)	30 (0.10)	63 (0.21)	147 (0.51)
6	9	23,600 (82.4)	0 (0)	35 (0.12)	148 (0.51)
14	13	27,500 (61.2)	147 (0.32)	122 (0.27)	320 (0.71)
13	16	37,500 (83.2)	110 (0.24)	19 (0.04)	130 (0.29)
15	16	36,000 (80.0)	40 (0.09)	70 (0.15)	42 (0.09)
16	16	39,000 (86.8)	55 (0.12)	42 (0.09)	73 (0.16)
18	16	38,500 (85.5)	71 (0.16)	64 (0.14)	65 (0.23)
11	20	43,000 (95.6)	0 (0)	0 (0)	152 (0.34)
8	21	22,000 (76.7)	255 (0.89)	0 (0)	570 (2.0)

SUMMARY

1. Radioactivity following intravenous injections of benzpyrene-5-C^{14} can be recovered quantitatively. No radioactivity appears in the respiratory CO_2. The compound and its metabolites are rapidly excreted into the bile, gastrointestinal tract, and feces. There is significant absorption into body fat.

2. The half-time of disappearance of radioactivity from benzpyrene-5-C^{14} from the site of subcutaneous injection in tricaprylin is $1\frac{3}{4}$ weeks. Excretion takes place through the feces.

3. The rate of disappearance of radioactivity from the site of a single application of benzpyrene-5-C^{14} has two phases, the half-times of which are approximately $1\frac{2}{3}$ days and $4\frac{1}{3}$ days.

4. These samples have been processed into the following fractions: neutral, acidic, loosely bound, and tightly bound to protein.

5. The significance of these results has been discussed.

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Inhibition of 9,10-Dimethyl-1,2-benzanthracene Skin Carcinogenesis in Mice by Polycyclic Hydrocarbons*†

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The addition of 1,2,5,6-dibenzofluorene to methylcholanthrene delays the appearance of tumors induced by this carcinogen in the skin of mice (2, 3). Neither chrysene, fluorene, nor 1,2,7,8-dibenzofluorene has this delaying action on methylcholanthrene carcinogenesis (3).

In the present series of experiments, a number of hydrocarbons were tested for their power to delay or to prevent the appearance of skin tumors in mice treated with 9,10-dimethyl-1,2-benzanthracene. This was done by comparing the effects of the carcinogen alone with the effects of a mixture of the carcinogen and test substance.

The compounds[1] selected for testing were: (I) a group of hydrocarbons of simpler structure than the carcinogen: naphthalene, anthracene, and fluorene; (II) 1,2-benzanthracene and three of its monomethyl derivatives: 2′-, 7-, and 8-methyl-1,2-benzanthracene; (III) 6,8-dimethyl-1,2-benzanthracene and 1,2,5,6-dibenzofluorene. Each of the compounds in Groups II and III was tested also for carcinogenicity under conditions comparable to its use as inhibitor.

MATERIALS AND METHODS

The test animals were CAF_1 hybrid mice about 4 months old. They were caged in groups of ten and received Rockland Mouse Diet (Arcady Farms Milling Co.) and water ad libitum. Twenty mice (ten males and ten females) were used in each experiment in which compounds of Groups I and II were tested. In each experiment with compounds of Group III, 30 mice (20 males and 10 females) were used.

Mineral oil[2] was employed as the solvent for all chemicals. The concentration of the carcinogen was 0.2 per cent,[3] and the concentration of the possible inhibitor was 0.4 per cent.[3] One drop of the test solution was applied twice a week with a glass rod to the interscapular skin, which was kept free of hair by clipping with scissors.

Applications of the carcinogen alone or of mixtures of the carcinogen and possible inhibitors were continued until the animals died or were killed. Animals were killed only when death was imminent and there was danger of loss of histological material.

Compounds in Groups II and III were tested for carcinogenicity by painting with a 0.4 per cent[3] solution twice weekly for the same length of time required to produce tumors in 100 per cent of animals receiving a mixture of 9,10-dimethyl-1,2-benzanthracene and the inhibitor. Application of the compound was then discontinued, and the animals were observed for a total of 68 weeks from the beginning of the experiments. In Group II, 2′- and 7-methyl-1,2-benzanthracene were applied for 33 and 32 weeks, respectively. In Group III, 6,8-dimethyl-1,2-benzanthracene and 1,2,5,6-dibenzofluorene were applied for 37 weeks. Applications of 1,2-benzanthracene and 8-methyl-1,2-benzanthracene were exceptions to this procedure; these compounds were applied throughout the 68-week period.

Charts were kept of all tumors induced in the painted area to record the time of their appearance

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[1] The authors wish to express their appreciation to Professor Melvin S. Newman, Ohio State University, for his generous assistance in supplying them with substantial amounts of 2′- and 7-methyl-1,2-benzanthracene.

For the purification or synthesis of the remaining compounds, the authors wish to thank Dorothy L. Butler, John G. Burr, and John H. Seipel, who worked as graduate students in the Department of Chemistry on the Evanston campus.

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[2] Superla White Mineral Oil, No. 34, U.S.P. Heavy, Standard Oil Co., Indiana.

[3] The concentration is expressed as weight volume per cent.

and their number and growth. Autopsies were performed on all mice, and relevant histological examinations were made.

RESULTS

Latent Period of Tumor Induction

The mean latent period of tumor induction was calculated from the length of time required for the appearance of the first tumor in each animal in each experiment (Chart 1, Table 1).

Group I (Experiments 1–4).—The four experiments in this group were done simultaneously. All animals survived and developed tumors. When the carcinogen alone was applied (Exp. 1), its mean latent period of tumor induction was found to be 10.0 ± 0.2 weeks.[4] In Experiments 2, 3, and 4, the effects of a mixture of the carcinogen with either naphthalene, anthracene, or fluorene were observed. The mean latent period obtained by use of the mixture of the carcinogen with naphthalene or with fluorene is not significantly different from that of the carcinogen alone. The differences are 0.4 ± 0.5 and 0.2 ± 0.3 weeks, respectively.[4] When anthracene was added to the carcinogen (Exp. 3), the mean latent period was 1.4 ± 0.4 weeks longer than that for the carcinogen alone.

Group II (Experiments 5–9).—These five experiments were done simultaneously. The calculations were based on the data on nineteen mice in four of the experiments because of the death of one mouse in each experiment before it developed a tumor.

In Experiment 5 the application of the carcinogen alone resulted in tumors with a mean latent period of 13.7 ± 0.5 weeks. The addition of 1,2-benzanthracene or its monomethyl derivatives to the carcinogen greatly delayed the appearance of tumors. 1,2-Benzanthracene prolonged the latent period by 10.7 ± 1.2 weeks; 2′-methyl-1,2-benzanthracene, by 9.5 ± 0.9 weeks; 7-methyl-1,2-benzanthracene, by 9.3 ± 0.9 weeks; and 8-methyl-1,2-benzanthracene, by 14.6 ± 1.2 weeks.

Group III (Experiments 10–12).—The three experiments in this group were done simultaneously. In Experiments 10 and 12, the calculations were based on the data on 29 mice because of the death of one mouse in each experiment before it developed a tumor.

The mean latent period of tumor induction for the carcinogen alone was 13.0 ± 0.3 weeks. The

[4] In each experiment the mean is given with its standard error, and the difference of means with the standard error of the difference. In each experiment the *t* test (1) was applied to differences in means and the value of P was determined.

P was 0.01 or less when the mean latent periods in Experiments 3, 6, 7, 8, 9, 11, and 12 were compared with the means of the corresponding control experiments (Exp. 1, 5, or 10).

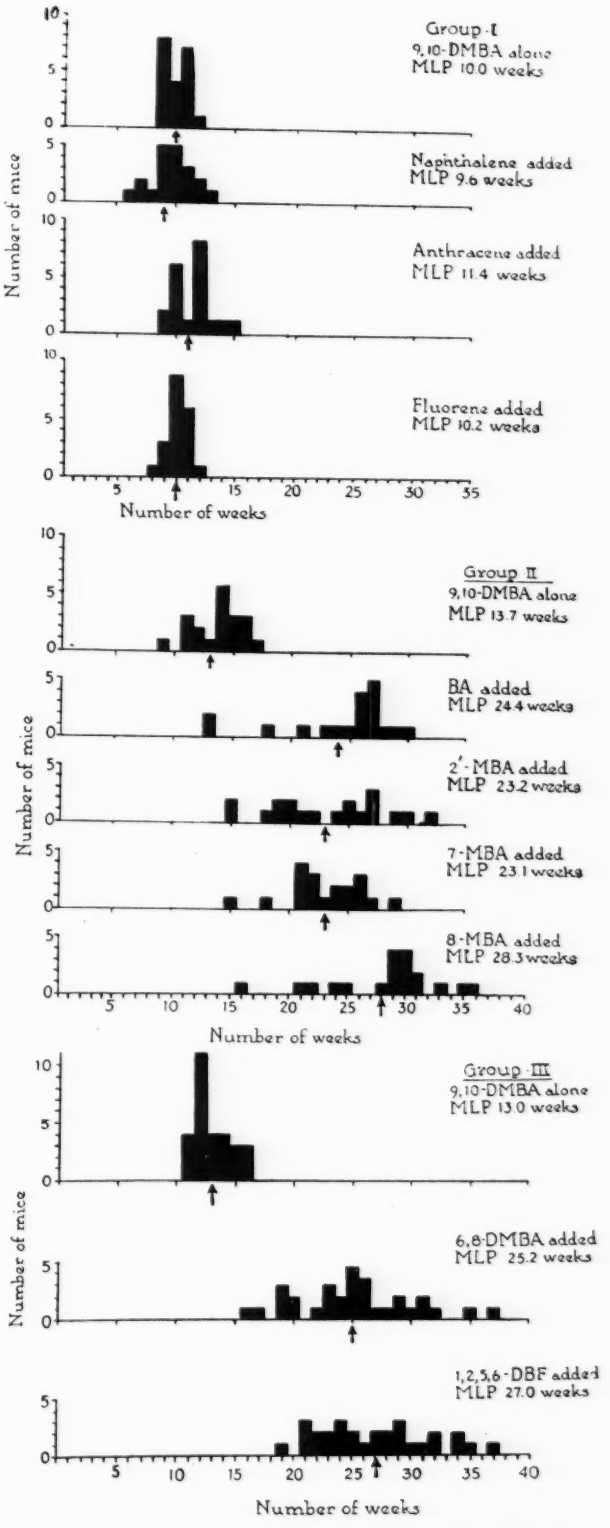

CHART 1.—The latent period of induction of the first tumor in mice receiving applications of the carcinogen 9,10-dimethyl-1,2-benzanthracene alone or in a mixture with a possible inhibitor. Arrows indicate means. MLP is mean latent period.

addition of 6,8-dimethyl-1,2-benzanthracene or 1,2,5,6-dibenzofluorene to the carcinogen greatly delayed the appearance of tumors; the former prolonged the latent period by 12.2 ± 1.0 weeks and the latter by 14.0 ± 1.0 weeks.

Total Number of Tumors Produced

The mean tumor yield per animal was calculated in each experiment (Chart 2, Table 1). The tumor count was based on the number of grossly recognizable tumors. This number often reached its maximum several weeks before the death of the animal, since, terminally, tumors frequently became fused or were replaced by ulceration or by extension of adjacent tumors. Occasionally, separate tumors regressed, but this was uncommon. Tumors that regressed were included in the tumor count if they had been present for at least 2 weeks. The data on five mice which bore one or more tumors but died prematurely of accident or infection were excluded from the calculation. The remainder of the mice died of tumor or were killed because death from tumor was imminent.

The tumor yield was not appreciably affected by the addition of the compounds in Groups I and II to the carcinogen, even though compounds in Group II did prolong the latent period significantly. In Group III, however, the mean tumor yield was decidedly less in those animals receiving a mixture of the carcinogen with 6,8-dimethyl-1,2-benzanthracene or with 1,2,5,6-dibenzofluorene (Exp. 11 and 12) than in those receiving the carcinogen alone (Exp. 10). In Experiment 10 the mean tumor yield was 8.6 ± 0.6. In Experiment 11 the tumor yield was 3.4 ± 0.8 less than in Experiment 10, and in Experiment 12 the tumor yield was 4.5 ± 0.7 less than in Experiment 10.[5]

[5] P was 0.01 or less when the mean tumor yields in Experiments 11 and 12 were compared with that in Experiment 10.

TABLE 1

LATENT PERIOD OF TUMORS, TUMOR YIELD, AND DURATION OF LIFE AFTER DEVELOPMENT OF TUMORS IN MICE RECEIVING CARCINOGEN OR MIXTURE OF CARCINOGEN AND INHIBITOR

Compounds	No. of mice in exp.	No. of survivors at first tumor*	Mean latent period† (weeks)	No. of mice dying of tumor‡	Mean tumor yield per mouse†	Mean duration of life after first tumor† (weeks)	Mean duration of life after fatal tumor† (weeks)
Group I:							
Exp.							
1. 9,10-Dimethyl-1,2-benzanthracene	20	20	10.0±0.2	20	12.3±1.1	13.4±0.8	10.1±1.0
2. 9,10-DMBA, Naphthalene mixture	20	20	9.6±0.4	20	14.6±1.7	13.2±1.2	9.5±1.1
3. 9,10-DMBA, Anthracene mixture	20	20	11.4±0.4	20	12.8±1.2	11.2±0.8	8.2±0.6
4. 9,10-DMBA, Fluorene mixture	20	20	10.2±0.2	19	14.6±1.1	12.2±0.5	10.3±0.6
Group II:							
Exp.							
5. 9,10-Dimethyl-1,2-benzanthracene	20	20	13.7±0.5	20	9.6±0.7	9.0±0.8	8.1±0.8
6. 9,10-DMBA, 1,2-Benzanthracene mixture	20	19	24.4±1.1	19	8.7±0.9	9.7±1.1	8.5±0.7
7. 9,10-DMBA, 2′-Methyl-1,2-benzanthracene mixture	20	19	23.2±1.1	19	8.5±1.0	8.8±0.7	8.1±0.7
8. 9,10-DMBA, 7-Methyl-1,2-benzanthracene mixture	20	19	23.0±0.8	18	7.2±0.8	9.3±0.7	7.4±0.5
9. 9,10-DMBA, 8-Methyl-1,2-benzanthracene mixture	20	19	28.3±1.1	17	8.4±0.7	10.1±0.6	8.9±0.6
Group III:							
Exp.							
10. 9,10-Dimethyl-1,2-benzanthracene	30	29	13.0±0.3	28	8.6±0.6	8.2±0.4	7.2±0.4
11. 9,10-DMBA, 6,8-Dimethyl-1,2-benzanthracene mixture	30	30	25.2±0.9	30	5.2±0.5	10.2±0.8	9.4±0.6
12. 9,10-DMBA, 1,2,5,6-Dibenzofluorene mixture	30	29	27.0±0.9	29	3.9±0.4	9.2±0.7	8.6±0.7

* All "survivors at first tumor" developed tumors.

† The data are listed with their standard errors.

‡ As indicated here and in the text, five tumor-bearing mice died prematurely of causes other than tumor.

DURATION OF LIFE AFTER DEVELOPMENT OF THE FIRST TUMOR

The duration of life after the appearance of the first tumor was determined for each animal, and the mean duration of life was then calculated from these data (Table 1). As in the estimation of mean tumor yield, the data on five tumor-bearing mice which died prematurely of causes other than tumor are excluded from the present calculation. In each group of experiments the duration of life after the development of the first tumor was approximately the same in animals receiving the carcinogen alone as in those receiving the mixture of carcinogen and inhibitor.

An estimate was also made of the mean duration of life after the appearance of that tumor (not necessarily the first) which later displayed the most malignant properties, i.e., that tumor which was most invasive or destructive and seemed to contribute most to the debility or death of the animal. In making this estimate, it was sometimes difficult to determine precisely which among a tight cluster of tumors of different ages had developed the malignant character. Subject to this error, the value for the mean duration of life after the appearance of a fatal tumor was not significantly different in animals receiving the carcinogen alone from that in animals receiving a mixture of carcinogen and inhibitor.[6]

The presence of an inhibitor evidently did not affect the progression of tumors once they had made their appearance.

CARCINOGENICITY OF THE INHIBITOR

As described in the section "Materials and Methods," the compounds of Groups II and III were tested for the possibility that they were themselves capable of inducing tumors under the conditions of the experiments (Exp. 13–18, Table 2). The animals in these tests were observed for a total of 68 weeks. 1,2-Benzanthracene induced one papilloma after 56 weeks of continuous painting, 2′-methyl-1,2-benzanthracene induced a papilloma 21 weeks after the cessation of a 33-week painting period, and 6,8-dimethyl-1,2-benzanthracene induced a papilloma 21 weeks after the cessation of a 37-week painting period. In the final week of a 37-week period of painting, 1,2,5,6-dibenzofluorene induced one papilloma; and in the following 31 weeks of observation four other mice developed one papilloma each. No tumors appeared during 32 weeks of painting with 7-methyl-1,2-benzanthracene and the subsequent 36 weeks of obser-

[6] Comparison of mean duration of life in animals receiving a mixture of carcinogen and inhibitor with the mean of those receiving carcinogen alone yielded a value of P greater than 0.01 in every instance.

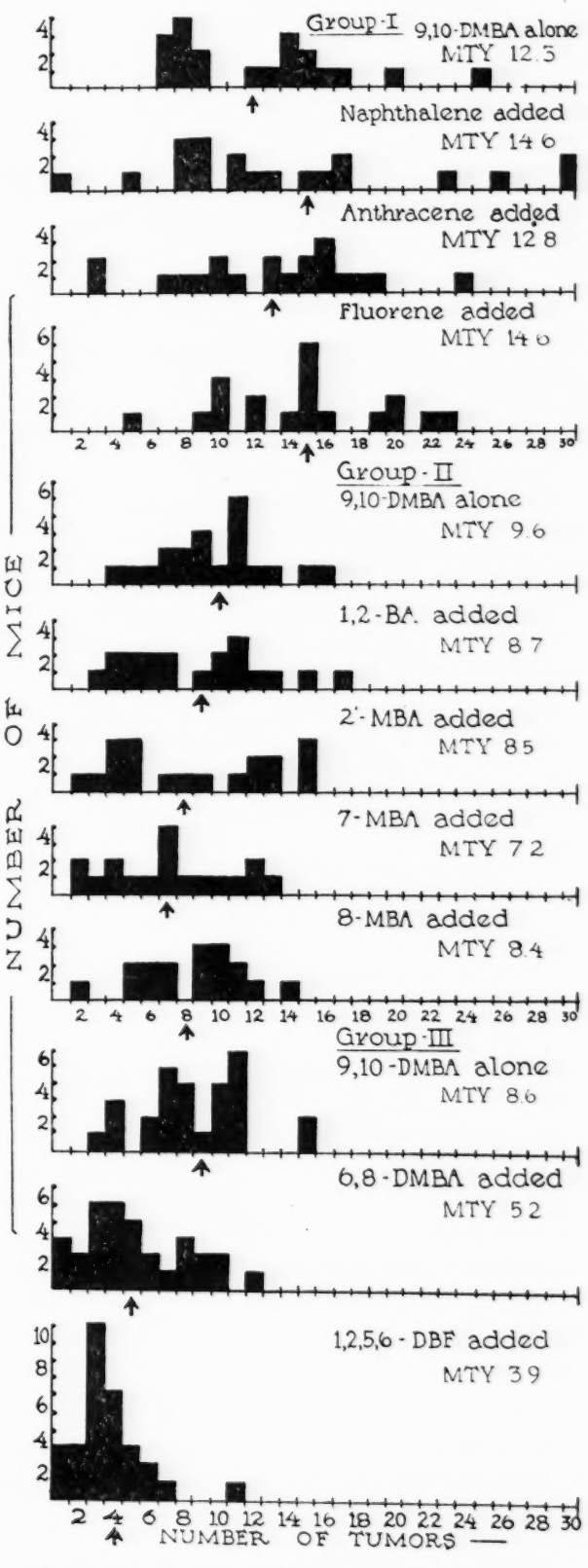

CHART 2.—The tumor yield per animal in mice receiving applications of the carcinogen 9,10-dimethyl-1,2-benzanthracene alone or in a mixture with a possible inhibitor. Arrows indicate means. MTY is mean tumor yield.

vation, or during 68 weeks of continuous application of 8-methyl-1,2-benzanthracene.

Factors of Sex and Weight

Males and females reacted similarly in all experiments.

The animals were weighed at intervals during the experiments and at death. There were no significant differences in weight in the animals in any of the experiments before the onset of tumors. After the development of tumors, of course, the weights of individual animals tended to vary considerably according to the size of the tumors and the degree of debility which they caused.

The most striking inhibition resulted from the addition to the carcinogen of the substances in Group III: 1,2,5,6-dibenzofluorene and 6,8-dimethyl-1,2-benzanthracene. The former prolonged the mean latent period of tumor induction by 107 per cent and reduced the mean tumor incidence per mouse to 42 per cent of the control. The 6,8-dimethyl-1,2-benzanthracene prolonged the latent period by 94 per cent and reduced the tumor incidence to 60 per cent of the control. Of the compounds in Group II, 8-methyl-1,2-benzanthracene had the strongest inhibitory effect, prolonging the mean latent period by 106 per cent. Prolongation of latent period by 1,2-benzanthracene and by the two other monomethyl benzanthracenes averaged about 70 per cent. Rudali *et al.* (4) have also reported that 1,2-benzanthracene has inhibitory action on skin carcinogenesis by 9,10-dimethyl-1,2-benzanthracene. Unlike the compounds in Group III, however, none of the Group II inhibitors significantly reduced the mean tumor yield. In Group I, which consisted of simpler polycyclic hydrocarbons, only anthracene showed any inhibitory properties. It prolonged the latent period by 14 per cent. Neither anthracene, naphthalene, nor fluorene affected tumor incidence significantly.

TABLE 2

Test of Carcinogenicity of the Inhibitors in Groups II and III

Compound	No. of mice in experiment	Duration of painting (weeks)	No. of survivors at first tumor	No. of mice with tumors at 68 weeks	No. of tumor-free survivors at 68 weeks
Group II:					
Exp.					
13. 1,2-Benzanthracene	20	68	15	1*	14
14. 2′-Methyl-1,2-benzanthracene	20	33	19	1†	17
15. 7-Methyl-1,2-benzanthracene	20	32		0	14
16. 8-Methyl-1,2-benzanthracene	20	68		0	14
Group III:					
Exp.					
17. 6,8-Dimethyl-1,2-benzanthracene	30	37	25	1‡	22
18. 1,2,5,6-Dibenzofluorene	30	37	30	5§	17

* This tumor appeared in the 56th week.
† This tumor appeared in the 54th week.
‡ This tumor appeared in the 58th week.
§ These 5 tumors appeared in the 37th, 50th, 51st, 58th, and 64th weeks.

Autopsy and Histological Examinations

Tumors from 256 of the 264 tumor-bearing animals in Experiments 1 through 12 were examined histologically. At least one of the tumors was an invasive squamous-cell carcinoma in every animal, except in the five tumor-bearing mice that died prematurely. In nineteen animals the tumors had metastasized to lymph node or lung.

DISCUSSION

A number of polycyclic hydrocarbons which are rather closely related to 9,10-dimethyl-1,2-benzanthracene were studied for possible inhibitory action on the induction of tumors by this carcinogen. It was found that two of the compounds prolonged the latent period of tumor induction and reduced the tumor yield and that certain others delayed tumor induction but did not affect the number of tumors produced.

Investigation of the carcinogenicity of the inhibitory compounds failed to produce unequivocal evidence of a relationship between carcinogenic and inhibitory power. The compounds of Group I, naphthalene, anthracene, and fluorene, have never been shown to be carcinogenic. Of these three,

anthracene showed slight inhibitory power. The compounds in Groups II and III were tested for carcinogenicity under conditions similar to those under which they inhibited tumor production. The two substances in Group III which, as inhibitors, both prolonged the latent period and reduced the yield of tumors were weakly carcinogenic. Of the four substances in Group II which, as inhibitors, prolonged the latent period but did not affect tumor yield, two were weakly carcinogenic and two failed to induce tumors. One of the latter two, 8-methyl-1,2-benzanthracene, was the most effective inhibitor in the group. Tests of the inhibitory power of other substances, including potent carcinogens, are in progress.

The mode of action of these inhibitory hydrocarbons remains unknown. The number of tumors induced in these experiments may be an index of the tumor-initiating power of 9,10-dimethyl-1,2-benzanthracene and the length of the latent period may be an index of its promoting power. If this is true, the compounds in Group III reduced both the initiating and promoting capacities of the carcinogen, while the compounds in Group II and, perhaps, anthracene in Group I interfered only with its tumor-promoting power. This and other possible interpretations of the effects of inhibitors on the initiation and promotion of tumors require experimental confirmation.

There is as yet no evidence that the inhibition is of a specific type. It may be that prolongation of latent period and reduction of tumor yield in these experiments are simply two manifestations of a relatively nonspecific cellular injury by the inhibitors. Such injury might or might not affect most severely those cells that have been altered in the direction of neoplasia. On the other hand, any more general injurious activity exercised by these inhibitory substances when mixed with potent carcinogens is not manifest either in the rate of growth and progression of established tumors or in the health and longevity of the treated animals.

Approaches to an understanding of the inhibitory mechanisms now under investigation include the local effects of the inhibitory substances, the relation of degree of inhibition to concentration of carcinogen and inhibitor, and the inhibition of carcinogenesis produced by administration of substances by means other than painting of the skin.

SUMMARY

1. The admixture of 6,8-dimethyl-1,2-benzanthracene or of 1,2,5,6-dibenzofluorene prolonged the mean latent period and reduced the mean yield of tumors induced in the skin of mice by application of 9,10-dimethyl-1,2-benzanthracene.

2. The mean latent period was prolonged, but the mean tumor yield was not reduced by admixture of 1,2-benzanthracene, or of 2′-, 7-, or 8-methyl-1,2-benzanthracene. Anthracene caused slight prolongation of latent period.

3. Naphthalene and fluorene had no inhibitory effect.

4. The progression of established tumors and the mean duration of life after development of a tumor were not significantly affected by mixture of any of the compounds with 9,10-dimethyl-1,2-benzanthracene.

5. 1,2-Benzanthracene, 2′-methyl-1,2-benzanthracene, 6,8-dimethyl-1,2-benzanthracene, and 1,2,5,6-dibenzofluorene were weakly carcinogenic when tested for carcinogenicity under conditions comparable to those under which they caused inhibition.

ACKNOWLEDGMENTS

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Announcements

The American Association for Cancer Research, Inc.

ANNUAL MEETING

The annual meeting of the American Association for Cancer Research, Inc., will be held in New York City on April 11–13, 1952.

Abstracts of papers to be presented at the meetings will be published and distributed before the meeting. The abstracts must be submitted to the chairman of the program committee, *Dr. S. P. Reimann, The Institute for Cancer Research, 7701 Burholme Avenue, Fox Chase, Philadelphia 11, Pennsylvania*, **not later than January 5, 1952.**

Abstracts submitted by nonmembers must be introduced by a member of the Association.

No paper will be placed on the program unless an adequate abstract is submitted. The abstract must not exceed 250 words in length. The style used in previous abstracts published in *Cancer Research*, **11**:233-92 (April) 1951, is to be followed. The title should appear as desired on the program. Following it should be the name of the author or authors and his or their university, laboratory, or hospital. Abstracts must be typewritten, double-spaced, on $8\frac{1}{2}\times11$-inch paper, and submitted in triplicate.

Further information concerning the meeting will be sent directly to the members of the American Association for Cancer Research, Inc., by the Secretary.

Grants for Scholars in Cancer Research

Announcement is made of grants for American Cancer Society Scholars in cancer research. A limited number of American Cancer Society Scholars are appointed annually on recommendation of the Committee on Growth of the National Research Council. A grant of $18,000, payable over 3 years, will be made directly to each Scholar's institution by the American Cancer Society as a contribution toward his support or his research or both.

Medical Schools, hospitals, research institutes, and other institutions with a primary or substantial interest in cancer research are invited to submit applications for these grants. Applications for grants to be effective July 1, 1952, should be submitted prior to January 1, 1952. Inquiries or requests for application forms should be addressed to the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.

Inquiry regarding Damon Runyon clinical fellowships, formerly recommended by the Committee on Growth, should now be addressed to the Damon Runyon Memorial Fund, Hotel Astor, Broadway and 44th Street, New York 19, New York.

Exchange Fellowships in Cancer Research

British American Exchange Fellowships in Cancer Research of the American Cancer Society, awarded by the Society on recommendation of the Committee on Growth of the National Research Council, are offered to citizens of the United States for advanced training and experience in Great Britain in specialized fields of investigation pertaining to cancer. Similar fellowships are awarded by the British Empire Cancer Campaign to British scientists for study in the United States. Fellowships are open to citizens of the United States who possess the degree M.D., Ph.D., or Sc.D. Applications should state the institution where the fellow plans to work in Great Britain; the individual under whom the fellow desires to work; what problem he intends to investigate, and when he wishes to start. Fellowships will be awarded for 1 year, and the annual stipend will be $4,020.00. An allowance of $600.00 is made for travel to the site of the fellowship in Great Britain. University staff appointment, with teaching duties agreeable to the fellow, is permitted, provided it is acceptable to the Committee on Growth, the American Cancer Society, and the British Empire Cancer Campaign. No other remunerative work will be permitted during the tenure of the fellowship. Application forms for British American Exchange Fellowships in Cancer Research may be procured from and submitted to Dr. O. M. Ray, Executive Secretary of the Committee on Growth, Division of Medical Sciences, National Research Council, 2101 Constitution Avenue, Washington 25, D.C. Fellowships will be made effective at the convenience of the institution and the fellow.